# STUDIES ON CONNECTIVE TISSUE GROUND SUBSTANCE

I. *Histochemical Features of Ground Substance Mucopolysaccharides*

II. *Organization of the Ground Substance in Ascorbic Acid Deficiency and its Modification by the Action of Cortisone*

BY

BENGT H. PERSSON

ALMQVIST & WIKSELLS BOKTRYCKERI AB
UPPSALA 1953

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BENGT H. PERSSON

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# Introduction

The functional properties of the connective tissue proper are intimately related to the reactivity of its ground substance. Though extensively studied from chemical, physiological and morphological points of view, the functional significance of this complex substance remains partly obscure.

The first part of this work deals with the histochemical features of the highly molecular acid mucopolysaccharides which constitute the carbohydrate part of the ground substance. This subject has frequently been considered by the histologists but few and inconsistent data are available on the reactivity of the isolated and purified constituents of the carbohydrate moiety of the ground substance.

The mucopolysaccharides possess a certain degree of plasticity of their physico-chemical constitution and it seems justified to assume that this property brings about a varying reactivity of the substances towards the reagents used to indicate their presence within the tissues. The main object of the first part of this work was to get more information on this problem.

The second part of the work deals with effect of ascorbic acid deficiency on the organization and reactivity of the ground substance. In addition the influence of cortisone on the ground substance in scurvy has been studied. Ascorbic acid as well as cortisone are known to induce significant changes in the connective tissues. Curiously enough, the structural changes brought about by ascorbic acid deficiency are in many ways similar to those found in hypoadrenalism as well as after administration of the adrenocortical hormone cortisone. Nevertheless, several workers have noticed a curative effect of cortisone on the scorbutic lesion in connective tissues. The purpose of the use of cortisone in the present work was to test its effect on the disintegrated ground substance found in scorbutic guinea pigs.

# I

# Histochemical Features of Ground Substance Mucopolysaccharides

CHAPTER I

# On the histology of loose connective tissue

The following orientation forms a short survey of the constituents of the so-called loose connective tissue. For a detailed description of this subject the reader is referred to modern histological manuals.

The loose connective tissue may serve as the prototype of the various connective tissues found in the animal organism. It contains almost all the cellular and intercellular elements which build up the more specialized varieties. The relative preponderance of either one of these elements constitutes the main basis for the classification of connective tissues in general.

The loose connective tissue is a direct derivative of the mesenchyme and may be considered as a rest of this embryonic tissue which is left when all the other, more specialized types have been formed (Maximow, 1927). Accordingly it has many potentialities and can develop into almost any mesodermal tissue. It is widely distributed in the organism, principally in the subcutaneous, submucous and subserous tissues, filling out the spaces between the organs and penetrating into them as membranes or sheaths.

The components of the tissue include cellular elements such as fibroblasts, macrophages and mast cells, and the extracellular elements—collagenous, reticular and elastic fibers and *the amorphous ground substance* or matrix. These elements are mixed up in various proportions and structural arrangements depending on the site and actual function of the tissue.

In an ordinary oversight-stained section from a tissue sample of the skin or some other organ rich in loose connective tissue, little or no ground substance can be seen. There are, however, seemingly empty spaces between the fibers which become more prominent when the tissue is distended with fluid. It is in these spaces that the ground substance exists. The relative amount of this material varies with age. In embryonic mesenchyme there are large quantities of ground substance, and it maintains its relative preponderance in loose connective tissue during the early life of the animal organism. With increasing age the ground substance diminishes in amount, and in the aging organism there is often very little to be found.

The nature of the matrix was earlier a matter of dispute among histologists. Virchow (1852, 1859) stated that at least in some types of connective tissues the amorphous matrix had a mucous character.

Later, other investigators came to the same conclusion (Hansen, 1899, 1908; Björling, 1911; Laguesse, 1914, 1921; Maximow, 1927; Clark and Clark, 1933; Bensley, 1934, and others). Nageotte (1922) on the other hand, expressed the opinion that there was only a "tissue fluid" circulating between the cells and fibers. Recent investigations have made probable that *the ground substance is a structurally organized material and that its most significant components are of a mucoid character* (Gersh and Catchpole, 1949).

Intermingled with the ground substance there is probably a varying amount of "tissue fluid", which is related primarily to the plasma and contains proteins, crystalloids, metabolites, etc. The existence of a *freely movable fluid* in normal connective tissue proper is however denied by McMaster and Parsons (1939, 1950). They state that though there undoubtedly exist large amounts of interstitial fluid, which is independent of the ground substance, this fluid is present in a captivated state in the form of perifibrillar films attached to the connective tissue fibrils.

It is generally accepted that the ground substance is a product of secretion by connective tissue cells. The exact source of the ground substance production is, however, not certain. The fibroblasts have been implicated by several workers, often only on the strength of their appearance in regions of excessive matrix production. More convincing reasons in support of such a theory are given by Bensley (1934), Gersh and Catchpole (1949), Holmgren (1949) and Mancini *et al.* (1950). Bensely, as well as Gersh and Catchpole, report morphological changes in the fibroblasts in areas of repair with an excessive production of ground substance, the cells showing signs of secretion of small granules from their cytoplasm. Holmgren was able to demonstrate the appearance of newly formed ground substance in fibroblast tissue cultures.

Other workers have made the mast cells responsible for the production of the ground substance (Aykroid and Zuckerman, 1938; Sylvén, 1940, 1941; Asboe-Hansen, 1951), or at least of part of it. It seems reasonable to conclude that *the origin of the ground substance is still not fully clarified.*

## Physico-chemical features of the ground substance

The ground substance itself is a complex material. Its physico-chemical nature is not fully defined. This is explained by the obvious fact that there are no specific extraction procedures for the matrix *per se*. The material extracted from connective tissues contains a mixture of substances, the origins of which are indeterminable. Chemical extraction of certain tissues rich in intercellular substance (vitreous body, umbilical cord, skin, synovial fluid) has revealed the presence

of acid mucopolysaccharides in association with protein (Meyer *et al.*, 1934, 1936, 1939, 1941; Blix, 1940). There are probably several varieties of acid mucopolysaccharides in the ground substance of different tissues. At present five different mucopolysaccharides are being distinguished in connective tissue: hyaluronic acid, a monosulfate ester of hyaluronic acid, and three types of chondroitin sulfuric acid (Meyer, 1950). From skin, as a source of loose connective tissue, two of these substances have been isolated, namely, hyaluronic acid and one of the types of chondroitin sulfuric acid (called chondroitin sulfate B by Meyer, 1950). It has been proposed that these two components exist in about equal amounts in fresh human skin (Pearce and Watson, 1949).

While it is certainly beyond the scope of the present short survey to go into details of the possible chemical constitution of the acid mucopolysaccharides, some statements on this problem, which are at present commonly accepted, may be mentioned here.

According to Meyer (1945) *hyaluronic acid* is classified as a simple mucopolysaccharide containing N-acetylated glucosamine and glucuronic acid in equimolar portions which constitute a disaccharide unit. Such units are polymerized to long chain structures containing about 70 disaccharide units in the molecule (Blix, 1951). According to Blix the molecule is mainly linear but the structure may vary in hyaluronic acids from different connective tissue sources. The exact molecule weight of hyaluronic acid is not known, but it is probably very high. Hyaluronic acid occurs either in a dissolved form as in synovial fluid or in the form of gels, as in umbilical cord and dermis (Meyer, 1947). Preparations from various sources show no significant physico-chemical differences except for viscosity (Meyer, 1952). The varying viscosity found in preparations from different sources points to polydispersity of the hyaluronic acid. Under physiological conditions the mucopolysaccharide is supposed to occur as an easily dissociated salt essentially free from protein. At low pH hyaluronic acid forms salts with proteins which may precipitate either as a mucin clot or as floccules, depending on the actual degree of polymerization of the hyaluronate (Meyer, 1947). Ropes *et al.* (1947) and Ogston and Stanier (1950) propound another opinion concerning the protein-hyaluronic acid relationship in the tissues. They state that protein may be firmly associated with hyaluronic acid even at physiological pH.

The second carbohydrate component isolated from loose connective tissue, *chondroitin sulfuric acid* (ChS), is classified, according to Meyer's scheme, as a complex acid mucopolysaccharide. It is supposed to possess a long chain structure built up by N-acetylated galactosamine sulfuric acid and glucuronic acid in equimolecular amounts. The molecular weight estimations have led to values of about 250 000 (Blix and Snellman, 1945). The physical properties of native ChS and the nature

of the complexes in which they may occur in the matrix are very difficult to ascertain. Thus far ChS has not been obtained in an undegraded form and it is never as viscous as hyaluronate.

The nature of the association between protein and ChS is a matter of dispute. Blix (1940, 1945) states that there is no indication of the presence in connective tissues of basic proteins which could form salts with polysaccharide acids at physiological pH. Meyer (1952) on the other hand claims that ChS in skin and in umbilical cord is firmly bound to proteins which are distinct from collagen. A salt-like linkage between collagen and ChS is, however, suggested to be present in cartilage.

The data concerning the chemistry of the ground substance complex are mainly concentrated on the carbohydrate moiety of the substance. Very little is known about the nature of the protein components of the matrix. Ultraviolet absorption spectroscopy of homogenized rat tail tendons has demonstrated the presence of a protein which is distinct from collagen (Lisco, 1949). As revealed by electrophoretic studies on synovial fluid, this "ground substance" contains several proteins with different mobilities (Blix, 1940; Hesselvik, 1940). The proteins of the ground substance moiety are generally attacked by trypsin, as has been shown by purification of extracted mucopolysaccharides (Meyer, 1945; Ropes *et al.*, 1947 and others). A destructive action of this enzyme on the ground substance of skin and fascia has been reported by Day (1947), Gersh and Catchpole (1949) and Gross (1950).

## Concepts of the structural organization of the ground substance

The ground substance of connective tissue is believed to exist in a gel-like state (Clark and Clark, 1933; Bensley, 1934; McMaster and Parsons, 1939; Gersh and Catchpole, 1949). This property is related to the mucoid components i. e. carbohydrate-protein complexes which by aggregation may form submicroscopical structures (Gersh and Catchpole). It is suggested that these structures are orientated and condensed and, under certain circumstances, constitute the microscopically visible basement membranes. These membranes are interposed between a cellular surface and the underlying connective tissue.

In the polymerized state, the ground substance is relatively inert chemically and the reactivity of the substance is said to increase when depolymerization takes place (Gersh, 1951). Evidence has been brought forward by histological and physiological research in support of the general assumption that *the ground substance gel exhibits a pronounced plasticity*. This characteristic forms the basis for the functional qualities of the ground substance.

The structural orientation of the matrix seems to be related to the fibrillar elements penetrating the substance. Studies on the mode of spreading of colored matter within connective tissue have revealed that the fibrils are surrounded by a thin fluid layer bordered outwards by "sheaths" of ground substance gel (McMaster and Parsons, 1950).

Unlike most histologists Day (1947, 1949, 1952) states that the interfibrillar substance belongs essentially to the class of structured proteins. These are seen in the dark field microscope as extremely thin membranes containing very delicate, branching fibrils. Day recently advanced the theory that the highly molecular mucopolysaccharides aggregate and act like stoppers impacted in the meshes of the protein net. *Thus the submicroscopical organization of the ground substance would mainly depend on the orientation of its protein components.*

Weiss (1933, 1949) suggests that the ground substance plays an essential role in organ differentiation in that it provides a patterned substrate formed by molecular orientations in the matrix. He has furnished experimental support for this concept by studying the growth of cells in a gelled matrix. The cell growth brings out an orientation in the matrix by presenting pathways of least resistance to the migrating cells.

Weiss' theory points to a direction that would seem to be essential in every discussion of the physico-chemistry and/or the microscopical and submicroscopical organization of the ground substance, that is, the assumption of the connective tissue in general, and of the ground substance in particular, as being dynamic. This implies that the physical and chemical state of the ground substance, as well as its structural organization, varies from time to time depending on functional conditions. This has been stressed especially by Gersh and Catchpole (1949). It has also been evident in many works on the action of hormones and of other factors on the connective tissue (Duran-Reynals, 1942, 1950). This "plasticity" of the ground substance seems to be intimately related to the action of the connective tissue cells. These are proposed to produce the essential mucopolysaccharide-protein complexes as well as some depolymerizing enzymes of the mucinase class (Meyer, 1947). The most well-known of the mucinases acting on the ground substance is the hyaluronidase. Its action on the acid mucopolysaccharides in connective tissues has been made the subject of an enormous literature. This research work will not be discussed here as there are excellent reviews by Duran-Reynals, 1942, Meyer, 1947, and Gibian, 1951, to which the reader is referred.

A few statements on the action of hyaluronidase on the loose connective tissue matrix may, however, be summarized here for bearing relation to the following discussion on the histochemical properties of the ground substance.

The hyaluronidases are enzyme complexes found in animal tissues

such as testes and skin. The enzymes are even found in various bacteria such as streptococci and pneumococci and elsewhere. Hyaluronidases from different sources vary significantly in their substrate specificity and in their mode of action (Meyer *et al.*, 1940; Humphrey, 1946).

Testicular hyaluronidase as well as hyaluronidases from other sources evidently contain several enzymes having depolymerase and hydrolytic activity (Hahn, 1946; Rogers, 1948). Some of these enzymes exert an action even on chondroitin sulfuric acid (Meyer *et al.*, 1940; Madinaveitia and Stacey, 1944; Hahn, 1947). According to Meyer *et al.* (1950) this is not due to the existence of a special enzyme in testicular preparations but to the same enzyme that acts upon hyaluronic acid. Bacterial hyaluronidases produced by pneumococci and streptococci do not attack chondroitin sulfuric acid.

The action of hyaluronidases on their main substrate, hyaluronic acid, is mainly twofold, i. e. a depolymerization with a lowering of the viscosity and, secondly, a cleavage of the molecules with a release of reducing substances. The depolymerizing effect is a rapid one making the substrate unable to form complexes with proteins at acid pH (mucin clot). Hyaluronic acid loses its gel character and becomes fluid.

It may be added that the hyaluronic acid gel is depolymerized when acted upon by many non-enzymatic substances such as ascorbic acid and some of its oxidation products (Robertson *et al.*, 1940; McClean and Hale, 1940; Skanse and Sundblad, 1943; Daubenmerkl, 1951).

It is suggested that the greater part of the hyaluronidases present in the animal organism is stored in a bound, inactive form and that only a small part exists in the free, active state (Mayer, 1950).

Hyaluronidase inhibitors are present in blood as well as in other tissues. The activity of these inhibitors fluctuates depending on functional or pathological conditions. (For further details the reader is referred to the review by Gibian, 1951.)

*Summary.* — The ground substance is an optically homogeneous material, the nature of which is essentially colloidal. The most characteristic components of the substance are the highly molecular acid mucopolysaccharides, hyaluronic acid and chondroitin sulfuric acid, which are more or less firmly linked to protein of an essentially unknown nature.

Normally the ground substance exists in a gel-like state which is related to the aggregation of the mucopolysaccharide-protein complexes, which may form submicroscopical structures. The structural organization of the ground substance is supposed to be closely related to the orientation of its protein components.

The ground substance of loose connective tissue is attacked by proteolyctic as well as by mucolytic enzymes.

CHAPTER II

# Demonstration of ground substance in tissue sections

In the following a review is given of the histochemical methods commonly used to demonstrate the ground substance within connective tissue sections.

*Metachromasia.* — The metachromatic reaction is characterized by the staining of certain tissue components in a color different from that of the dye solution itself. The term "metachromasia" originates from Ehrlich (1879) who called the shade of the dye solution itself *orthochromatic* as distinguished from the different *metachromatic* coloration given to certain tissue elements by the same single dyestuff. The substances exhibiting metachromatic staining properties are commonly called *chromotropic* (or metachromatic) substances.

Among the dyes exhibiting metachromatic properties those most frequently used are the *thiazins* including such dyestuffs as thionin, toluidine blue and the azures. All these are basic dyes showing a blue orthochromatic color and a violet or reddish metachromatic shade.

The physico-chemical nature of metachromasia is still a matter of dispute. (For detailed surveys of the literature on this special problem reference is made to Zeiger (1938), Sylvén (1941), and Michaelis and Granick (1945).) Lison (1935) was the first to study metachromasia from a physico-chemical point of view. He introduced the term "true metachromasia" which means that the metachromasia is resistant to alcohol treatment. According to Lison a true metachromasia is highly specific to sulfuric esters of high molecular polysaccharides, like chondroitin sulfuric acid or heparin. This interpretation is shared by Holmgren and Wilander (1937), Sylvén (1941) and many others. Later investigations, however, have shown that the specificity of the reaction is not bound to the existence of sulfuric acid groups in the molecule of the substrate but probably to a certain density of negatively charged groups (Bank and Bungenberg de Jong, 1939; Wiame, 1947). In later years many different substances have been shown to be chromotropic.

Of special interest in this connection is the metachromasia produced by the ground substance and its relation to the known acid mucopolysaccharides of this substance. As pointed out already by Lison (1933), chondroitin sulfuric acid shows a strong metachromatic reaction with tiazines. Regarding the metachromatic property of hyaluronic acid, different opinions have been advanced in the literature. Thus Meyer (1947) states that purified hyaluronic acid in a concentration of at

least 1 per cent shows a metachromatic reaction with toluidine blue. After Meyer several workers suggested that hyaluronic acid is metachromatic in various tissues. There are others who deny this property of hyaluronic acid (Davies, 1943, 1952).

*Basophilia.* — The ground substance as a whole has been characterized histologically as a *basophilic* "structure", i. e. a material with a certain affinity to basic dyes. This is dependent on the acidic groups of the components constituting the matrix. The basophilia of the mucopolysaccharides within the matrix has been investigated by several workers (Dempsey *et al.*, 1946, 1947, 1950; Laves, 1948; Pearse, 1949, 1950). These investigations are based on those made by Pischinger (1926, 1927) and Zeiger (1930) who used the basophilic property to determine the isoelectric point of various tissue structures. By spectrophotometrical measurements of the capacity of various carbohydrate-protein complexes to bind methylene blue at various pH, Dempsey *et al.* found that while glycoproteins begin to take up the dye at pH 6, the acid mucopolysaccharides show this property already at pH 4 or less. It must be noted that the authors give no analytical data on the test substances used.

Hempelmann (1940) suggests a method to differentiate various mucoproteins by making use of their different affinity to toluidine blue in extreme solutions or by adding proper amounts of ethyl alcohol to moderately diluted solutions of the dye. The intensity of the metachromasia and the orthochromatic shade given to certain structures were then estimated visually. Hempelmann states that this staining method allows of a differentiation between epithelial and mesenchymal mucoproteins in the tissues. No studies on isolated, chemically verified substances have been reported by that author.

*Affinity to metals.* — The affinity of the acid jelly of the matrix to heavy metals has been potined out long by Bensley (1934) who reported that the ground substance had a certain affinity to copper salts. No technic was outlined for the histochemical use of this property. In 1946 Hale devised a method where he made use of colloidal ferric hydroxide linked to the acidic groups of the mucopolysaccharides (carboxyl and sulfuric acid groups). The adsorbed iron is demonstrated as Prussian blue by treatment with hydrochloric acid and potassium ferrocyanide. According to Hale the iron does not combine with proteins or neutral polysaccharides. However, the specificity of the reaction is very uncertain. Lillie and Mowry (1949) as well as Pearse (1951) have shown that the binding of iron is by no means an ability of acid mucopolysaccharides only. Hale advises treatment, before staining, with streptococcal hyaluronidase (which is entirely specific to hyaluronic acid) in order to distinguish hyaluronic acid from other blue-staining structures.

The specificity of the method is thus limited by the purity of the enzymes used.

*Aldehyde reaction.* — The action of periodic acid on polyalcohols was described by Malaprade in 1928. According to him, the periodic acid attacks the hydroxyl groups of α-glycols, splits the carbon-carbon linkage and oxidizes the hydroxyls to aldehydes. The aldehydes can then be demonstrated by certain reagents like Schiff's leucofuchsin. A similar mode of action of periodic acid on polysaccharides was stated by Jackson and Hudson (1938). Nicolet and Shinn (1939) were able to show that even if one or both of the hydroxyls are replaced by amino-groups or alkylamino groups, the reactivity of the compound to periodic acid remains unaltered.

The histochemical adaptation of the periodic acid oxidation followed by Schiff's reagent was suggested by McManus (1946) and, independently, by Hotchkiss (1948). In a matter of a few years, this has now become a technic very often used in staining carbohydrates within tissue sections. After the basic work of Hotchkiss on the histochemical *periodic acid—Schiff reaction (hereafter called PAS),* this reaction has been further explored by Pearse (1949), Lillie (1950) and Gomori (1952). Pearse notes that besides glycogen and certain sugar-containing lipoids a positive result indicates mucopolysaccharide, mucoprotein or glycoprotein. Lillie points out that a positive reaction is given by collagen, gelatine and reticulin, sometimes by coagulated plasma, serous exudates and fibrin. He suggests a return to the original interpretation of the Malaprade reaction, without inferring mucopolysaccharide structure. The interpretation of the histochemical specificity of the PAS-reaction in other than connective tissues is not discussed in the present work.

Of special interest in this connection are the divergencies reported in the literature concerning the PAS-reactivity of hyaluronic acid. Thus Hotchkiss states that the acid reacts strongly positive to the PAS-reagents. This finding is confirmed by Jorpes *et al.* (1948) who also showed heparin monosulfuric acid to give a strong positive reaction. Jeanloz (1950), on the other hand, points out that purified hyaluronic acid consumes very little periodic acid for each repeating unit, a finding which was independently made by Blix (1951).

The second main mucopolysaccharide in connective tissues, the chondroitin sulfuric acid, is said not be PAS-positive (Hotchkiss; Jorpes *et al.*). Nevertheless, it has been reported several times in the literature that the ground substance of cartilage, which is known to contain large amounts of this acid but no hyaluronic acid, takes a more or less heavy color with the PAS-method. It would therefore seem that there are some unexplored divergencies between the statements made by the chemists and the findings made by histochemical

application of the PAS-reaction. In this connection one must remember the existence of fibrillar elements within the mucopolysaccharide gel. Collagen and reticulin show a positive PAS-reaction (Wislocki *et al.*, 1948). Hydroxyproline, one of the main protein components of collagen, has been shown to reduce periodic acid (Carter and Neville, 1947). Furthermore, other amino acids like serine, threonine and hydroxylysine might react (Lillie, 1950). On the other hand Hotchkiss (1948) as well as Gomori (1952) state that the amino acids in question are tied up by peptide linkages that block their reactive groups within the tissues.

To sum up, the PAS-reaction as prescribed by Hotchkiss may be considered *a common reaction of all potential aldehydes which are unmasked by the action of periodic acid.* In fixed animal tissues freed from lipoids this reaction is assumed to demonstrate the presence of glycogen, mucoproteins, glycoproteins, glycolipids and phospholipids. The validity of the reaction for demonstrating the existence of certain acid mucopolysaccharides within the ground substance is not fully elucidated and requires further exploration.

Some trials have been made with the use of other oxidizing agents replacing the periodic acid. Lothka (1952), Glegg and Clermont (1952) report the use of lead tetraacetate which was shown by Criegee (1940) to attack the same groups as periodic acid and, in addition, α-oxy acids. So far no spectacular results seem to have been gained by this method.

*Digestion with enzymes.* — The action of hyaluronidase has been widely used as a complement to various staining methods to ascertain the presence of hyaluronic acid presumably made visible by the staining method employed. While it must be emphasized that this is the only method available at present to suggest the presence of hyaluronic acid in certain tissue structures, the need for careful evaluation of the results obtained in such a way has not always been duly realized in the works published. Not only is the question concerning the substrate specificity of utmost importance in this connection (Laves, 1948), but there is also the possibility of a non-specific blocking action af the enzyme molecules on the surface of the substrate, an action which impedes the penetration of the enzyme into the interior of the section (Danielli, 1947).

*Discussion and summary.* — Of the various methods used to reveal the nature and location of the ground substance within connective tissues none can be said to be entirely specific either with regard to the matrix as a whole or to the defined constituents of the complex.

The following conclusions may be derived from a critical examination of the data reported on the reliability of the histochemical methods available for the demonstration of the ground substance complex or

part of it in microscopical tissue sections: The ground substance complex may be visualized in tissue sections treated in a proper way with metachromatic staining, with the periodic acid-Schiff technic, as well as with the aid of its affinity to iron salts.

The reactivity of the various ground substance constituents towards the different reagents has, however, not been properly defined. This seems to be due to lack of experimental data on the staining properties of the constituents when tested in a purified form.

Though it has been noted that the stainability of the ground substance varies with different conditions of the connective tissue, few conclusive data are available concerning the relation between the physico-chemical state of the ground substance components and their actual stainability with the various reagents. To obtain such data *in vivo* is of course very difficult, if at all possible. It seems justified, however, to make an attempt to elucidate the problem by employing purified substrates isolated from connective tissue as "models", using histochemical procedures which resemble, as closely as possible, those used on ordinary tissue specimens.

## Object of the present investigations

This study represents an attempt to estimate the stainability of purified acid mucopolysaccharides isolated from connective tissue sources by the methods mentioned above. Special stress has been laid on the study of the reactivity of hyaluronic acids from different sources towards the various reagents.

The chromotropic potency of hyaluronic acids has been specially investigated. Already at the beginning of these studies it became evident that the commercial metachromatic dyes available, showed very prominent variations of their metachromatic properties. This suggested that the dyes possessed a varying degree of purity. Thus, part of the work reported is concerned with the purification of metachromatic dyes and the analyses of the purified stains obtained with respect to their metachromatic properties.

CHAPTER III

# Own Investigations

## Chromatographic separation of colored materials in commercial batches of toluidine blue o and azure I

Toluidine blue and azur I are chemically closely related salts of the basic thiazin group which consists of one phenyl and one quinone group joined by a sulfur atom.

Commercial batches of the dyes actually show very different metachromatic properties when tested on chromotropic substances or tissue structures. The metachromatic shade obtained with various dye samples differs both in color and intensity; in some instances one gets a distinct metachromasia in a certain structure of a tissue section, whereas, upon duplication of the staining with the use of another sample of supposedly the same dye, entirely different results will be obtained. This is especially obvious when staining weakly metachromatic structures. It seemed reasonable to assume that these findings were a result of differing purity of the dye batches.

As it was my intention to study the metachromatic properties of purified acid mucopolysaccharides and to make an attempt to characterize these properties with the use of spectrophotometrical data, the first step was to get reasonably pure dyes.

### Materials and methods

Five batches of toluidine blue o (C. I. 925) and three of azure I (C. I. 923), all of which had been used in the laboratory for metachromatic staining purposes, were tested histologically and chromatographically. Of the five toluidine blue batches two were Grübler dyes, one of which had been stored in the laboratory for about ten years (marked 9—42). Of the other three, two were made in Germany and one in England. Of the three azure samples two were certified dyes

from Coleman and Bell in the U.S.A. and one was an old batch made in Germany.

To test the *staining properties* of the various commercial dye batches, serial sections from an 18-day-old rat foetus were stained for 30 minutes in 0.05 % aqueous solutions of the dyes. The stained sections were differentiated in 96 % alcohol for exactly 2 minutes and dehydrated in absolute alcohol for 2 minutes, whereafter the sections were mounted in balsam.

*Adsorption chromatography* was carried out on columns of alumina oxide (for chromatography acc. to Brockmann) in Pyrex glass tubes 3 cm wide and about 50 cm in height. The adsorbent was packed dry with mechanical pressure up to three quarters of the glass tube. The lower end of the tube was connected to a suction bottle which was evacuated with an ordinary water suction pump.

After wetting the column with distilled water 500 mg of the dyestuff dissolved in 50 ml distilled water was added to the column. Once the dye solution had been added, the chromatogram was developed by adding additional quantities of distilled water until maximum development.

During the eluation the fractions passing the column and dropping into the suction flask were successively collected for spectrophotometrical studies.

After maximum development the column was dried by continued suction. The adsorbent was extruded from the glass tube and the dye bands were separated by cutting up the alumina with a spatula. Special care was taken to avoid mixing of adjacent bands by discarding the boundary zones.

Eluation of the sections was performed with methyl alcohol (or ethyl alcohol). The filtered solutions were evaporated to dryness at 60° C and in some cases weighed.

To control the uniformity of the main fractions obtained, corresponding fractions from two columns running with the same batch of dye were mixed and chromatographed again in a third column. Such controls were performed with one sample of toluidine blue and one of azure.

In some instances the dye fractions were controlled with respect to their uniformity by a simple type of partition chromatography using circular filter papers according to the method described by Rutter (1948). A mixture of butanol, glacial acetic acid and water (40 : 10 : 50) as prescribed by Partridge (1948) was used as solvent and the chromatograms were developed for 3 to 4 hours at room temperature.

*Spectrophotometrical investigations.* — The commercial dye batches, as well as the fractions obtained from them, were studied in a Beckman DU spectrophotometer in aqueous solutions or dissolved in ethyl

alcohol. The fractions collected from the suction bottle during development of the chromatogram were used after filtration, no attempt being made to estimate the concentration of the dye substance. 1 cm Corex cuvettes were used in all instances.

The metachromatic potency of the various dyes was estimated by adding constant amounts of reasonably pure chromotropic substances to the stains. The substances used were heparin (as representative of sulfate containing metachromatic substrates), sodium metaphosphate[1] (a polymerized salt reported to be metachromatic by Wiame, 1947) and polyacrylic acid[1], a high molecular carbohydrate, the negative charge of which is attributed to carboxyl groups only. Metachromasia produced by this substance has to my knowledge not been reported earlier.

To test the "negative metachromasia", i. e. a typical shifting of the maximum absorption towards the long wave lengths by the addition of sufficient amounts of nucleic acid (Michaelis and Granick, 1945; Lison and Mutsaars, 1950), a commercial sample of yeast nucleic acid was used.

The concentrations of the stains and test substances were identical in all the experiments performed and are indicated on the graphs shown in Figs. 1—4 (see pp. 26—27).

In order to facilitate further studies on the metachromatic potencies of hyaluronic acid and other test substances it was desirable to express the "degree of metachromasia" numerically. As proposed by Wiame (1947) this can be done by using the ratio between the extinction values of the metachromatic band (gamma-band) and the orthochromatic band (alpha-band). The ratio is low when determined on a highly diluted stain (in water) and becomes higher when chromotropic substance is progressively added to the dye. In the present work the term "*metachromatic index*" is adopted for the ratio of the extinction value at 550 mμ to that at 615 mμ. Polyacrylic acid dissolved at 0.714 mg per 100 ml of 1 mg % aqueous solution of the stain, is employed as chromotropic test substance.

## Results

The staining properties of the eight commercial dyes are shown in Table 1. Dyes B, C, D, and E may be regarded as "satisfactory metachromatic stains" with respect to their staining of the dermal ground substance (which has a low degree of chromotropic potency). The remaining four batches may be designated as "unsatisfactory metachromatic dyes".

[1] Kindly placed at my disposal by doc. B. Ingelman.

Table 1. *Staining Characteristics of Commercial Azure I and Toluidine Blue.*

| Dye | Cartilage | Mast cells | Ground substance (skin) | Nuclei |
|---|---|---|---|---|
| A. Azure I (M–k) . | Blue violet | Violet | Unstained–pale blue | Blue |
| B. Azure I (C–B) (Cert. Cz–3) ... | Violet | Red violet | Pale red violet | Blue |
| C. Azure I (C–B) (Cert. Cz–4) ... | Violet | Red violet | Pale red violet | Blue |
| D. Toluidine blue (G–r) ..... | Red violet | Red | Pale red violet | Blue |
| E. Toluidine blue (G–r) ..... | Red violet | Red | Pale red violet | Blue |
| F. Toluidine blue (M–k) ..... | Blue violet | Deep violet | Unstained–pale violet | Blue |
| G. Toluidine blue (G–rr) ..... | Red violet | Red violet | Pale blue violet | Blue |
| H. Toluidine blue. | Blue violet | Violet | Unstained–pale blue | Blue |

When fractioning the various dyes on the alumina columns all the dyes show a similar principal development. The first fraction leaving the column showed a green or rose color (Fraction 1). Second, a light blue band separated, followed by a dark blue one (Fraction 2). If the first fractions leaving the column are collected at short intervals, a number of samples may be obtained which show small but significant differences in their spectral absorption maxima.

At maximum development, the main part of the dye had separated into three rather sharply bounded fractions: A pale violet band on the top of the column followed by a broad, deep violet fraction (Fraction 3; see Figs 2 and 4). Below this fraction the column is colored blue (Fraction 2).

Obviously the relative amounts of the different fractions varied within the dyes. However, no attempts were made to determine the quantities of the fractions obtained and no data of value can therefore be given. So much may be said, however, that the yield of the major metachromatic fraction (Fraction 3) was higher from the "satisfactory dyes" than from the "unsatisfactory" ones.

Though the fractions obtained were visually similar in the two dye groups, they showed important differences when studied histologically and spectrophotometrically. These differences will be seen from Table 2 (p. 28) and Figs. 1—4 (pp. 26—27).

As seen from the graphs in Figs. 1 and 3 the commercial dyes (whole dyes) from both groups show similar absorption curves with two or three absorption peaks within 590 to 630 mμ. Curves of such a type suggest the possibility of mixtures of dyes.

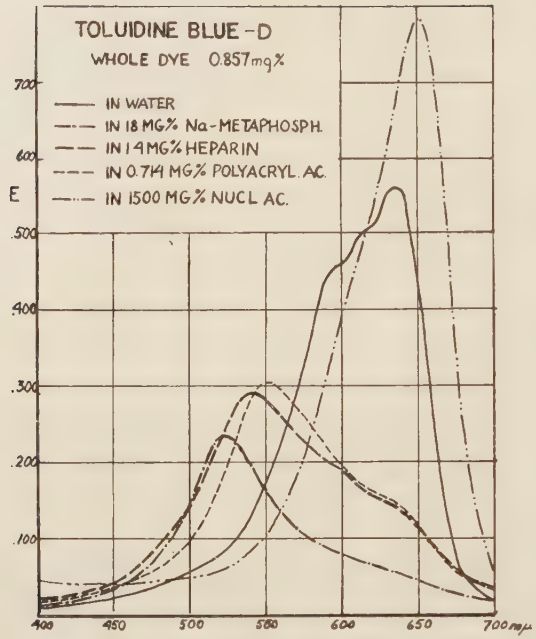
TOLUIDINE BLUE - D
WHOLE DYE 0.857mg%
IN WATER
IN 18 MG% Na - METAPHOSPH.
IN 1.4 MG% HEPARIN
IN 0.714 MG% POLYACRYL. AC.
IN 1500 MG% NUCL. AC.
E
700
600
500
400
300
200
100
400
450
500
550
600
650
700 mμ

Fig. 1.

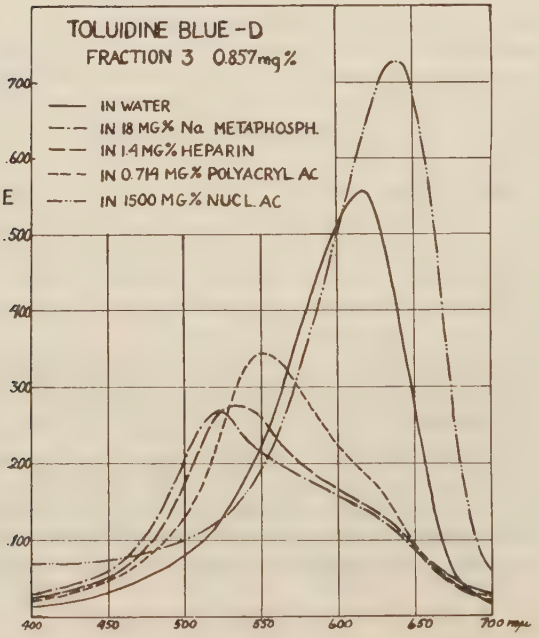
TOLUIDINE BLUE - D
FRACTION 3 0.857mg%
IN WATER
IN 18 MG% Na METAPHOSPH.
IN 1.4 MG% HEPARIN
IN 0.714 MG% POLYACRYL. AC.
IN 1500 MG% NUCL. AC.
E
700
600
500
400
300
200
100
400
450
500
550
600
650
700 mμ

Fig. 2.

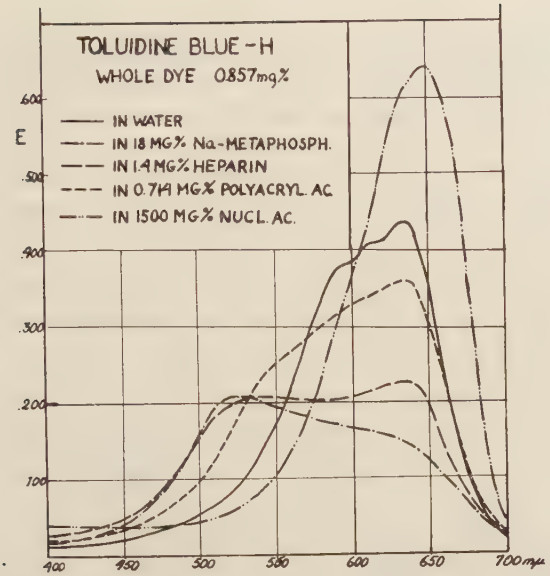
TOLUIDINE BLUE-H
WHOLE DYE 0.857mg%
IN WATER
IN 18 MG% Na-METAPHOSPH.
IN 1.4 MG% HEPARIN
IN 0.714 MG% POLYACRYL. AC.
IN 1500 MG% NUCL. AC.
E
.600
.500
.400
.300
.200
.100
400
450
500
550
600
650
700mμ

Fig. 3.

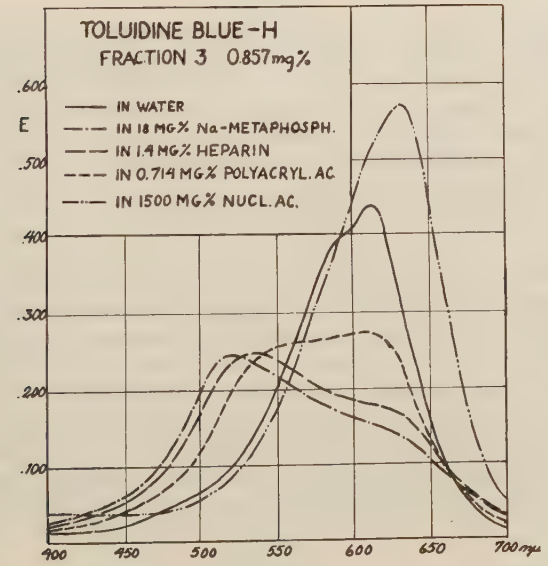
TOLUIDINE BLUE-H
FRACTION 3 0.857mg%
IN WATER
IN 18 MG% Na-METAPHOSPH.
IN 1.4 MG% HEPARIN
IN 0.714 MG% POLYACRYL. AC.
IN 1500 MG% NUCL. AC.
E
.600
.500
.400
.300
.200
.100
400
450
500
550
600
650
700mμ

Fig 4.

Table 2. *Metachromatic Potency in vitro ("Metachromatic Index") and Staining Properties of Representative Whole Dyes and Constituent Fractions.*

(The figures within brackets represent the ratio $E_{550} : E_{615}$ in pure water).

| Dye | Absorbtion maximum mμ | Metachromatic index $E_{550} : E_{615}$ | Visible metachrom. | Color of | | |
|---|---|---|---|---|---|---|
| | | | | cartilage | mast cells | ground sub |
| A. Whole dye | 600—640 | 1.06 (0.41) | + | Blue violet | Violet | No staini |
| Fraction 1.. | 595—660 | 0.77 (0.77) | − | No staining | No staining | No staini |
| Fraction 2.. | 610—640 | 0.74 (0.76) | − | Blue | Blue | No staini |
| Fraction 3.. | 600—615 | 1.29 (0.64) | + + | Violet | Violet | Pale viole |
| C. Whole dye | 610—640 | 1.40 (0.37) | + + | Violet | Red violet | Pale red vi |
| Fraction 1.. | 610—660 | 0.29 (0.30) | − | No staining | No staining | No staini |
| Fraction 2.. | 625 | 0.64 (0.21) | (+) | Pale blue | Pale blue | No staini |
| Fraction 3.. | 615 | 1.58 (0.58) | + + + | Red violet | Red | Pale red vi |
| D. Whole dye | 590—630 | 1.65 (0.35) | + + | Red violet | Red | Pale red vi |
| Fraction 1.. | 600—650 | 0.48 (0.49) | − | No staining | No staining | No staini |
| Fraction 2.. | 630 | 0.65 (0.63) | − | Pale blue | Pale blue | No staini |
| Fraction 3.. | 615 | 1.84 (0.43) | + + + | Red violet | Red | Brick red |
| H. Whole dye | 590—630 | 0.75 (0.41) | + | Blue violet | Violet | No staini |
| Fraction 1.. | 590—640 | 0.68 (0.71) | − | No staining | No staining | No staini |
| Fraction 2.. | 610—630 | 0.66 (0.63) | − | Deep blue | Blue | No staini |
| Fraction 3.. | 590—615 | 0.96 (0.53) | + + | Violet | Red violet | Pale viole |

The band located at the longest wave length is commonly called the alpha-band of the dye whereas the peak at the shorter wave length corresponds to the so-called beta-band (or dimeric band—Michaelis and Granick, 1945). In aqueous solutions the alpha and beta bands lie each at one definite wave length but their relative heights are changed when varying the dye concentration. The metachromatic band or gamma-band is displaced toward a shorter wave length; it is not visible in the spectral curve of an aqueous solution of the dye but appears when adequate amounts of a chromotropic substance are added to the dye solution. The metachromatic band is interpreted as due to high polymers of the dye molecules (Michaelis and Granick).

The spectral curves produced by the addition of the various chromotropic substances differ in the two dyes. This is particularly striking when polyacrylic acid is concerned. This difference is also reflected in the metachromatic indices given in Table 2 (dyes D and H). It may be mentioned that the indices of the dyes with low metachromatic potency were about 0.7 to 1.1 under the given conditions, whereas the indices of the satisfactory dyes reached 1.4 to 1.7.

The spectral curves of the metachromatic fractions (Fraction 3) of the dyes D and H (see Table 2) are shown in Figs. 2 and 4. As shown

in these graphs the maximum absorption of the metachromatic fraction of dye D is sharply located at 615 mμ, whereas the corresponding fraction from the "unsatisfactory" dye H has a broad absorption peak which possibly includes two or more constituents.

When chromatographing this fraction on an alumina column no further separation of the components of the dye was obtained. Other methods to separate the suggested sub-fractions were not tried.

The metachromatic potency of the two fractions is demonstrated by adding the same test substances as used with the whole dyes. As seen from the spectral curves and from Table 2 (metachromatic index), the metachromatic potencies of Fractions 3 are in both instances higher than those of the mother dyes. When examining the staining properties of the various fractions (see Table 2) one contaminating, non-metachromatic fraction of the unsatisfactory dyes stained various structures in a blue color of a rather high intensity. On the other hand, the corresponding fraction from the satisfactory dyes (B, C, D, and E) exhibited very poor, if any, staining properties.

To sum up, all the commercial dyes tested were composed of several constituents. At least one of them showed metachromatic properties of a varying quality. The contaminating dyes either possessed no staining action at all or stained various structures in a more or less intense blue (orthochromatic) color.

## Discussion

Although the chromatographic technic used in the present experiments is probably not ideal for a complete separation of the dye mixtures, this method may be suggested as being sufficient for obtaining reasonably pure dyestuffs. When used under standardized conditions, such purified dyes may give reproducible staining pictures with chromotropic structures within microscopical tissue sections. Obviously this is an asset to the histologist when estimating the amount of metachromatic material within the tissues. Moreover, purified dyes seem to be indispensable in any study of the metachromatic properties of isolated substances *in vitro* at least when reproducibility is a desideratum in the technic used. It must be emphasized however, that the possibility of making such chemically undefined staining reactions reproducible depends on several factors besides the purity of the stain used.

Concerning the influence of the contaminating, non-metachromatic fractions on the metachromatic potency of the dye mixture, it may be suggested that the fraction possessing a strong blue-staining property might obscure the color produced by the metachromatic fraction at least when the chromotropic potency of the substrate is low. Such an

effect may be partly responsible for the poor metachromatic properties of the dye sample H in the present studies. The effect of the non-staining colored materials (Fraction 1) on the staining properties of the metachromatic fraction(s) remains obscure. It may be added that this fraction often showed a more or less pronounced fluorescence in ultraviolet light, with a color varying from pale blue to green.

With respect to the purposed application, the metachromatic fraction obtained from the dyes with high metachromatic potency seems to be satisfactory. Judging from the spectral curve, this fraction may be considered homogeneous. Its staining properties were excellent with regard to the metachromatic as well as the orthochromatic shade. In my further studies this fraction (obtained from dyes D and E, see Table 1) was used in all the metachromatic stainings performed (in some instances completed with staining with the metachromatic cyanine dye pinacyanol).

Without any intentions of going into the intricate problem of the physico-chemical nature of metachromasia it may be pointed out that the absorption maxima obtained with the various chromotropic substances tested, differ significantly with respect to the location of the gamma peak (Figs. 1—4). Michaelis and Granick (1945) suggest that the strength of the displacement of the gamma-band toward violet is dependent on the dye concentration. According to the spectral data obtained in the present studies, the displacement may be influenced additionally by the nature of the chromotropic factor. The height of the gamma peak in relation to the alpha peak (i. e. the metachromatic index) seems to be at a maximum when a certain optimal amount of chromotropic agent is added to the dye solution. This will be further discussed below.

## The metachromatic properties of hyaluronic acid as compared to other acid mucopolysaccharides

As mentioned earlier the metachromatic reaction is one of the tools used to demonstrate the ground substance within connective tissues. The reaction is mostly described as being dependent on the existence of sulfuric acid esters of mucopolysaccharides—according to the statement made by Lison (1935).

Taking into account, however, that in certain locations the amount of hyaluronic acid isolated from the connective tissue constitutes at least one half (Pearce and Watson, 1949) of the total amount of acid mucopolysaccharides present, it may be justified to make an attempt to elucidate the possible action of hyaluronic acid as a chromotropic

factor in the ground substance complex. The information on this problem found in the literature presents no definite evidence as to whether hyaluronic acid is to be regarded as a metachromatic substance or not. Furthermore, no data have been reported from adequate histochemical investigations on the isolated and reasonably purified substance.[1]

With respect to the reported plasticity of the ground substance gel in the tissues it seems to be of a certain interest to study the stainability of hyaluronic acid in relation to its actual state of polymerization and/or aggregation.

To make histochemical studies on isolated acid mucopolysaccharides more applicable to the "native conditions" as they are proposed to exist in loose connective tissue, the interaction of proteins has to be taken into account. In the present studies an attempt has been made to gain some information on this problem by studying the staining reactions of purified mucopolysaccharides mixed up with serum and by investigating the histochemical properties of the pure substances when injected into dermal connective tissue of experimental animals.

## Materials and methods

Purified hyaluronic acid from three different sources and chondroitin sulfuric acid from nasal septa of cattle were used.[2] The polysaccharides were isolated as their sodium and calcium salts, respectively, and analyzed as to their content of amino sugar, nitrogen and sulfur (and in some instances with regard to their content of glucuronic acid).

In addition, a commercial sample of heparin (Vitrum, without preservative) and glycogen extracted from the rabbit liver were used. No analytical determinations were made on these substances.

The test substances are listed in Table 3. The figures of relative viscosity represent the values found on 0.1 % solutions of the various hyaluronates in 0.05 M phosphate buffer at pH 7 and 25° C.[3] The figures within brackets are the values of the relative viscosity found when the hyaluronates were dissolved in distilled water and determined at 37° C in ordinary Ostwald viscosimeters with a flow time for distilled water ranging between 33.6 and 37.7 seconds.[4]

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[1] Sylvén and Malmgren recently published a paper on this subject. This work is discussed below (see p. 39).

[2] The hyaluronic acids as well as the chondroitin sulfates were prepared by prof. Gunnar Blix, Head of the Institute of Medical Chemistry of the University of Uppsala, who generously placed them at my disposal together with the analytical data found in Table 3.

[3] Determinations made at the Institute of Medical Chemistry.

[4] Determinations made by the author.

Table 3. *Specimens of Hyaluronic Acid and Chondroitin Sulfuric Acid Employed for Histochemical Studies.*

| Sample | Source | Viscosity $n_r$ | Hexosamine % | Glucoronic acid % | N % | S % |
|---|---|---|---|---|---|---|
| Hyaluronic acid – GK .. | Vitreous body | (4.2) | — | — | — | 0 |
| Hyaluronic acid – H 33.. | Umbilical cord | 2.43 (5.0) | 40.52 | — | — | 0 |
| Hyaluronic acid – H 66.. | Umbilical cord | 4.22 (14.2) | 44.34 | — | 3.55 | 0 |
| Hyaluronic acid – H 62.. | Umbilical cord | 6.72 (17.4) | 42.97 | — | 3.15 | 0 |
| Hyaluronic acid – H 58.. | Mesodermal cyst | (1.5) | 44.68 | 41.74 | 4.04 | 0 |
| Chondroitin sulfate – NI | Nasal septum | — | 28.98 | — | 2.76 | 5.31 |
| Chondroitin sulfate – NII | Nasal septum | — | 30.3 | 35.03 | 2.58 | 5.92 |

The test substances listed have been used in three different ways:

a) as aqueous solutions added to stains in test tubes and studied spectrophotometrically;
b) as smears applied to microscopic glass slides and treated as ordinary tissue sections;
c) as aqueous solutions injected into the skin of experimental animals and studied after sectioning as ordinary tissue sections.

The interaction of proteins on the staining was studied after mixing some of the substances with freshly prepared rabbit serum.

*Test tube experiments.* — The metachromatic color produced in the test tubes was studied in a Beckman DU spectrophotometer one hour after mixing the chromotropic substance and the dye solution. The metachromatic index (as defined on page 24) was estimated for a series of concentrations of hyaluronic acid isolated from vitreous humor (Sample GK; see Table 3) and from umbilical cord (Sample H 62; see Table 3). In addition, similar determinations were made on chondroitin sulfuric acid (Sample N II; see Table 3) and commercial heparin. The dye employed was a batch of purified toluidine blue (Grübler) at a constant concentration of 0.857 mg per 100 ml.

To study the interrelationship of the state of polymerization and metachromatic property some hyaluronic acids which differed with respect to their relative viscosities were studied (see Tables 3 and 4). The individual samples were used at a concentration of 0.1 mg per ml. One of the highly viscous samples (H 66) was depolymerized by treatment with 0.1 N NaOH. After neutralization with 0.1 N HCl the hyaluronate was precipitated with alcohol, dissolved in distilled water, and the relative viscosity was estimated in Ostwald viscosimeters.

The determinations of the metachromatic index were made on three independent samples of each hyaluronic acid.

*Smears of purified mucopolysaccharides.* — This part of the study was carried out with three samples of hyaluronic acid (listed in Table 3 as H 33, H 62, and H 58), chondroitin sulfuric acid (Sample N I), commercial heparin (Vitrum), and glycogen extracted from rabbit liver. The substances were dissolved at 0.5 % in distilled water and either used in this form or as mixtures with rabbit serum. These mixtures were prepared by dissolving the hyaluronic acid solutions (0.5 %) in various amounts of serum in ratios of 1 : 5 to 1 : 20. Controls were prepared by mixing distilled water and serum in corresponding proportions.

A 5 % aqueous solution of gelatine was smeared out in a thin layer on one half of thoroughly cleaned microscopical slides. Before the gelatine film was dry, the test substances were put into it by writing figures in the film with ordinary pens. In this way several substances can be applied side by side to one single slide (see Fig. 6). On other slides 0.2 ml of the various hyaluronic acid-serum mixtures were spread over an area of about 2.4 × 3 cm marked on the glass slide.

After drying in the air for half an hour the smears were fixed in a mixture of equal parts of ethanol and methanol for 10 to 15 minutes (in some cases Carnoy's fluid was used as fixing agent), whereafter they were covered with a film of celloidin to prevent diffusion of the test substances during the staining procedures.

*Smears of native tissues.* — Vitreous body from cattle and synovial fluid of human origin were smeared out on glass slides and stained either in the fresh state or after fixing with ethanol-methanol or Carnoy's fluid (ethanol-chloroform-glacial acetic acid—6 : 3 : 1).

*Intradermally injected test substances.* — Hyaluronic acid (Sample H 33; see Table 3) and chondroitin sulfuric acid (Sample N I) dissolved in distilled water at a concentration of 0.5 % were injected intradermally in eight guinea pigs. 0.1 ml of the solutions was used in each bleb. The animals were killed 15 minutes and 4 hours after the injections, four animals each time. The skin areas containing the blebs were removed and fixed in ethanol-methanol 1 : 1 at + 2° C or frozen instantaneously in isopentane chilled with fluid air to — 165° C and dehydrated in vacuum at — 41° C or in ethanol-methanol mixture at the same temperature, according to the method described by the author (Persson, 1952). After embedding in paraffin and sectioning, staining was performed with toluidine blue as described below.

*Staining procedures.* — The model smears were stained with chromatographically purified toluidine blue (dye batch D; see Table 1) and with commercial toluidine blue (batch H) or azure I. The stains were prepared in distilled water at a concentration of 0.1 %. Certain sections of the intradermally injected mucopolysaccharides were stained

with *pinacyanol* (C. I. 808), a basic dye of the cyanine group of dyes with pronounced metachromatic properties first demonstrated by Proescher (1933). This dye was used either as an aqueous solution at 0.05 % or dissolved in equal parts of distilled water and propylene glycol at a concentration of 0.5 %.

After removing the paraffin and passing through alcohol and water staining was performed for 15 minutes. Follows differentiation of the staining in 96 % ethanol for exactly two minutes (with agitation of the fluid) and dehydration in absolute ethanol for two minutes. After clearing in toluol for 5 to 10 minutes the smears and sections were mounted in synthetic balsam (Permount).

*Treatment with testicular hyaluronidase.* — Model smears were treated for 1 to 4 hours at 37° C with a few drops of hyaluronidase (Hyalas, Leo[1])—dissolved in McIlvaine's citrate buffer (0.05 M; pH 6.2; containing 0.15 M NaCl) at a concentration of 20 V.R.U./ml.[2] Controls were incubated at the same time with buffer solution only. After a brief washing in distilled water the smears were taken to alcohol and were covered with a celloidin film whereafter they were stained as described above.

In addition, some smears were treated with inactivated enzyme under identical conditions.

## Results

*The metachromatic index.* — Fig. 5 shows the results of the spectrophotometrical determinations of the ratio of the extinction values at 550 mμ and 615 mμ for increasing concentrations of hyaluronic acid (Samples H 62 and GK; see Table 3), chondroitin sulfuric acid (Sample N I) and heparin (commercial sample) added to a 0.857 mg% toluidine blue solution (Fraction 3 of Sample D; see Table 1). In the figure the metachromatic index is plotted against concentration of the test substances.

As will be seen from the graph, the metachromatic index increases with an increasing concentration of chromotropic agents. At high concentrations of these substances this tendency is reversed and the metachromatic index decreases. This effect depends on a simultaneous increase of the extinction value at 615 mμ and a depression of the value at 550 mμ. The effect is especially obvious when the sulfated mucopolysaccharides are concerned (see Curves 1 and 2 in Fig. 5). Obviously the two hyaluronic acids tested show different properties

[1] Kindly placed at my disposal by A. B. LEO, Hälsingborg.

[2] 1 V. R. U. = the quantity of enzyme reducing the viscosity of a certain hyaluronate to 50 % in 10 mins. at 37.5° C.

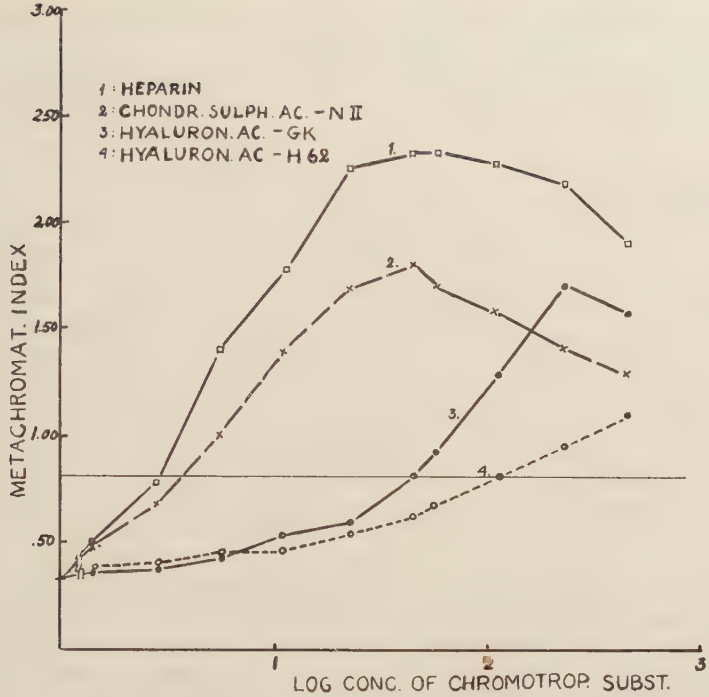


Fig. 5. Metachromatic index (ratio $E_{550} : E_{615}$) plotted against increasing concentrations of various chromotropic substances.

with respect to the ability to depress the metachromatic index at high concentrations. The hyaluronic acid GK (from vitreous humor) exerts the same effect on the metachromatic index as the sulfated polysaccharides (see Curve 3) when present at high concentration, whereas the sample isolated from umbilical cord (Sample H 62) show no such effect. The depression of the metachromatic index is followed by the formation of amorphous violet or reddish precipitates.

In Fig. 5 the horizontal line indicates the level where metachromasia becomes visually distinct. This level is naturally an arbitrary one, the position of which depends on several factors such as the wavelength of the transmitted light etc. In any case, it may roughly demonstrate the amounts of the different chromotropic agents necessary to produce a clearly visible metachromasia. Evidently a much higher concentration of the hyaluronic acids than of the sulfated mucopolysaccharides was required to produce a distinctly visible metachromasia. Under the present experimental conditions the typical change of color appears when the concentration of hyaluronic acid reaches a level of about 10 to 20 mg

Table 4. *Metachromatic Potency as Compared to Relative Viscosity of Hyaluronic Acid Samples.*

| Sample | Relative viscosity (0.1 % aqueous solution) | Metachromatic index: $E_{550} : E_{615}$ |
|---|---|---|
| Hyaluronic acid H 66 ..... | 14.2 | 1.09 (1.08—1.10)* |
| Hyaluronic acid H 66 — alkaline-treated | 3.1 | 0.66 (0.64—0.67)* |
| Hyaluronic acid H 66 — alkaline-treated | 1.5 | 0.60 (0.58—0.61)* |
| Hyaluronic acid H 33 ..... | 5.0 | 1.38 (1.37—1.40)* |
| Hyaluronic acid GK ..... | 4.2 | 1.30 (1.28—1.31)* |
| Hyaluronic acid H 58 ..... | 1.5 | 0.72 (0.70—0.73)* |

* Figures within brackets represent the limiting values determined.

per 100 ml. The corresponding values for the sulfated mucopolysaccharides are less than one tenth as high.

In Table 4 data are given on the metachromatic potency of various samples of hyaluronic acid exhibiting different relative viscosities.

Evidently the metachromatic index decreases with the relative viscosity, provided that one and the same sample is considered. This is clearly shown in sample H 66. Such an interrelationship is, however, not always to be found when testing different preparations of the mucopolysaccharide with respect to their viscosities only. As shown in the table two different preparations exhibiting comparable viscosity values may differ significantly with regard to their metachromatic potency. Thus the actual degree of polymerisation alone (as expressed by the relative viscosity) does not justify any predictions as to the metachromatic potency of the preparation concerned. The relatively high polymerized sample H 66 showed an unexpectedly low metachromatic potency as compared with the more fluid samples H 33 and GK. When depolymerized to a level comparable with that of these samples, the metachromatic potency of H 66 completely disappeared.

*Metachromatic reactions of dried smears of hyaluronic acid.* — When using satisfactory stains, dried hyaluronic acid smears showed a definite metachromasia, often with a quality comparable to that of chondroitin sulfuric acid. Heparin, however, showed a more reddish color than the other mucopolysaccharides. The metachromasia produced by the hyaluronic acid samples was no less stable towards alcohol treatment than was the color produced by the other polysaccharides. The various samples

of hyaluronic acid tested (H 33, H 62, and H 58; see Table 3) exhibited distinct differences in coloration with shades varying from blue violet (Sample H 58) to red violet (Sample H 33). Obviously the metachromatic properties were under these conditions the same as those reported from the test tube experiments described above.

When staining was performed with an unsatisfactory dye (as toluidine blue, Sample H; see Table 1) the hyaluronic acid smears were stained in an orthochromatic (blue) color, whereas the sulfated polysaccharides exhibited a violet, metachromatic color.

When hyaluronic acid was mixed with serum in proportions of 1 : 20 to 1 : 5, metachromasia was demonstrable up to a ratio of 1 : 15 when samples H 33 and H 66 were employed, whereas no metachromasia was obtained when sample H 58 (which showed a low metachromatic index *in vitro*) was mixed with serum in a ratio of 1 : 5. The metachromatic potency of the sulfated mucopolysaccharides does not seem to be depressed by the admixture of serum in these proportions.

The serum contained about 6.5 % protein (as estimated with the micro-Kjeldahl method). The actual concentration of hyaluronic acid in per cent of the dry weight of the mixture may thus be calculated as varying between 2 % (at a ratio of 1 : 5) and 0.4 % (ratio hyaluronic acid: serum = 1 : 20). *The lowest concentration of hyaluronic acid producing a definite metachromasia under the given conditions thus seems to be about 0.5 to 0.6 %.*

The results reported here are partly demonstrated in Fig. 6 showing some representative model smears stained with purified toluidine blue and with a commercial batch of the same dyestuff. The rather delicate differences in the shade of the metachromasia within the various smears cannot be demonstrated in an ordinary photo, but the differing intensities of the metachromatic reaction with the various substrates are clearly visible. Moreover, the figure conveys an idea of the prominent difference in the metachromasia obtained with the two stains used.

*The effect of testicular hyaluronidase on the model smears* was somewhat conflicting in view of the irregularity of its action on the different samples of hyaluronic acid. In the present experiments the hyaluronic acid showing the highest relative viscosity (Sample H 66) lost its metachromatic potency completely after one hour's treatment with the enzyme, whereas such an effect was established with respect to sample H 33 after an incubation for three to four hours. At that time the metachromasia of the chondroitin sulfuric acid had obviously weakened; but a similar effect was seen also in the controls and may thus be explained by the dissolving of the polysaccharide during treatment. Regarding sample H 58 (relative viscosity 1.5 in aqueous solution), the metachromasia of this sample was from the beginning of a low intensity and had completely disappeared after one hour's treatment with the hyaluronidase. Moreover, this depolymerized hyaluronic acid

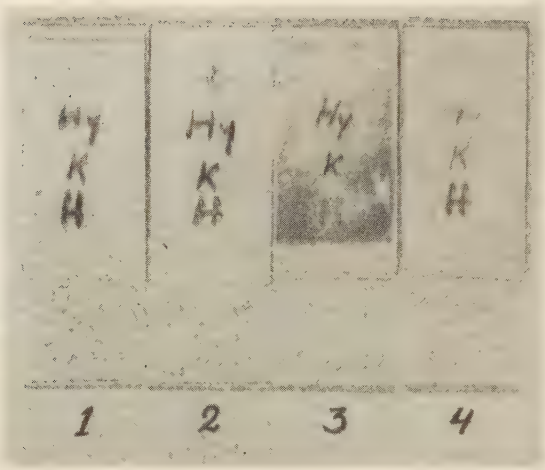


Fig. 6. Fixed smears of hyaluronic acid (Hy), chondroitin sulfuric acid (K)' and heparin (H).

1: stained with purified azur I (Fraction 3, dye B)
2: stained with purified toluidine blue (Fraction 3, dye D)
3: stained with commercial azur I (dye A)
4: stained with commercial toluidine blue (dye H).

seemed to be rather soluble in the buffer so that part of the substance disappeared from the gelatine film during the incubation procedure.

No effect similar to that produced by the active enzyme could be found when inactivated hyaluronidase was tested on the smears.

The samples of "*native mucopolysaccharide-protein complexes*" represented by vitreous body and synovial fluid both showed a positive metachromatic reaction when fresh smears on glass slides were covered with the stain solution. In the microscope it was possible to observe a progressive precipitation of a very delicate net of fibrils showing a brilliant red color. When treated with ethanol the metachromasia immediately disappeared from the vitreous body sample as seen macroscopically, but under the microscope some fibrils retained their red violet color and the "macroscopical metachromasia" reappeared when the alcohol was washed away with toluol. The metachromasia produced by the synovial fluid sample seemed to be stable against ethyl alcohol, at least parts of it which were microscopically visible as red precipitates.

Fixed smears of both substrates showed a definite, alcohol-resistant metachromasia bound to the precipitated fibrillar and membraneous structures.

According to the data reported in the literature the mucoid component

of synovial fluid as well as of vitreous body is hyaluronic acid (Meyer and Palmer, 1934; Meyer *et al.*, 1939). Synovial fluid is said to contain about 0.1 to 0.5 % of hyaluronic acid, and vitreous body about one tenth of this value. When dried or fixed in dehydrating agents the concentration of hyaluronic acid in these substances may exceed 10 per cent.

*Intradermally injected mucopolysaccharides.* — Hyaluronic acid as well as chondroitin sulfuric acid was fairly well visible within the skin sections 15 minutes after being injected. Both substances appeared as red violet floccules between the fibers of the dermal connective tissue when stained with the purified toluidine blue or with pinacyanol. The distinct metachromasia of the precipitates produced by these dyes differed significantly from the vague violet color obtained when staining was performed with the commercial dye batch H.

Four hours after injection no *metachromatic precipitates* were visible in the chondroitin sulfuric acid blebs. The presence of a chromotropic substance could however be discovered through the red violet color of the collagen within a circumscribed area of the section. Outside this area the collagenous fibers were practically unstained or faint blue.

At that time there still persisted metachromatically stained floccules of hyaluronic acid in some sections, whereas in others no traces of metachromasia could be observed. No metachromatic staining of the collageneous fibers like that described above was to be found in the hyaluronic acid blebs.

*Discussion.* — Evidently hyaluronic acids in a purified, sulfur-free form may possess a significant metachromatic potency comparable with that shown by chondroitin sulfuric acid. It is obvious, however, that the chromotropic activity of hyaluronic acids varies in different preparations showing comparable viscosities. This might be explained by differences in configuration of the molecules or molecular aggregates in accordance with the suggestion made by Blix (1951) that the structure of hyaluronic acid may vary in samples of different origin. The present experiments do not justify any inferences concerning the detailed physico-chemical conditions of the polysaccharide which may be relevant with respect to the observed differences in metachromatic potency.

The importance of using reasonably pure dyes in studies on metachromasia is clearly shown in the present investigations. It may be suggested that the divergencies found in the literature as to the metachromatic property of hyaluronic acid are at least partly accounted for by different metachromatic potencies of the dyes used.

In a recently published paper on the metachromasia of hyaluronic acid Sylvén and Malmgren (1952) propose that "most preparations

of hyaluronic acid from mesodermal sources are probably contaminated by sulfur-containing material". These authors gave close attention to the purity of the substrate but not to that of the dye. It may be mentioned that the commercial thiazin dye batches vary in their content of anhydrous dye salt between 50 % and 90 %. Moreover, in dye mixtures the actual dye content cannot be accurately determined (Conn, 1946). As shown in the present investigations all the dyes chromatographed were mixtures.

The metachromatic index as a measure of the chromotropic potency of acid mucopolysaccharides may be of value on the assumption that the spectrophotometrical determinations are performed under strictly standardized conditions with the use of a reasonably pure dye. The localization of the metachromatic gamma ($\gamma$) band produced by the substance concerned must be estimated beforehand. As pointed out by Michaelis and Granick (1945), as well as by Sylvén and Malmgren (1952), the position of this band is not a fixed one but may be influenced by the concentration of the dye and by the ionic strength of the solution. According to the data obtained in the present investigations each one of the chromotropic substances produces a gamma band with an absorption maximum that is more or less typical of the substance. This statement is probably valid only for substrate concentrations within certain ranges.

In the present studies the position of the peak of the metachromatic band was at 550 mμ when hyaluronic acid or chondroitin sulfuric acid were dissolved in purified toluidine blue solution. The orthochromatic alpha ($\alpha$) band of this dye had its absorption peak localized at 615 mμ. The maximum of the $\alpha$-band and that of the dimeric beta ($\beta$) band always lies in each case at one definite wave-length (Michaelis and Granick).

As shown in the present study (Fig. 5) there is an increase of the metachromasia (metachromatic index) with rising concentration of chromotropic agent. For the sulfated mucopolysaccharides and one of the sulfur-free hyaluronic acids this increase shows a maximum at certain concentrations of the substances whereafter the intensity of the metachromasia declines. Similar findings are reported by Wiame (1947) and by Levine and Schubert (1952). The decrease of the index was always accompanied by the appearance of metachromatically stained precipitates.

The data presented in Fig. 5 cannot be said to be fully correct with respect to the values obtained for heparin at higher concentrations of the substrate. The reason for this is the fact that the absorption maximum for the gamma band produced by this substance is not located at 550 mμ but at 535 mμ. This is of no importance when the concentration of heparin is low ; in that case the spectral curve shows no definite peak at 535 mμ but rather a plateau around 530 to 560 mμ. At high

concentrations, when the absorption peak is more pronounced, the values of the ratio $E_{550} : E_{615}$ do not adequately correspond to the high intensity of the metachromasia produced. These objections bear no relation to the other two mucopolysaccharides tested, for the position of their gamma bands is at 545—550 mμ.

The lowest concentration of partly depolymerized hyaluronic acid prepared from human umbilical cord giving a distinct metachromasia in dilute aqueous solution of purified toluidine blue was 20 to 40 mg per 100 ml. This is a rather low value in view of the data reported by other workers. Thus Meyer (1947) states that a concentration of hyaluronic acid—"highly enough polymerized"—of 1 % is required to produce a metachromatic reaction *in vitro*. Sylvén and Malmgren (1952) claim that "purified sulfur-free hyaluronic acid in concentrations below 1 to 2 per cent, corresponding to a sol state, does not produce absorption shifts typical of the metachromatic compounds". These authors used a depolymerized hyaluronic acid (particle weight about 60 000) obtained from umbilical cord. The divergencies between the findings in the present study and those of the cited workers may be assumed to originate from differences in the substrates as well as in the dyes used. *Thus it seems to be inaccurate to discuss the "metachromasia of hyaluronic acid" in general, for this property of the mucopolysaccharide is closely related to its actual physico-chemical state which may obviously vary in different preparations.*

Concerning the metachromatic potency of the various hyaluronic acid preparations in the form of smears, the same differences between the preparations were found as when they were studied in aqueous solutions. The lowest dry weight concentration of hyaluronic acid giving a distinct metachromatic reaction was calculated to be about 0.5 %. This value holds for one of the preparations, whereas another more depolymerized sample showed an orthochromatic staining at a calculated concentration of about 2 %. According to Sylvén and Malmgren dried films of purified hyaluronic acid mixed with gelatine with an average concentration above 1.8 % are metachromatic with Azure A.

In contradiction to the findings of Sylvén and Malmgren but in agreement with those reported by Wislocki *et al.* (1947) and Grishman (1948), fresh vitreous humor from cattle stained metachromatically with toluidine blue. The hyaluronic acid content in this tissue is about 48 mg per cent (Meyer, 1947). This value is comparable with that giving a metachromatic reaction in the test tube experiments discussed above.

A question of certain interest in this connection is this: Is it possible to transfer the findings made by studies of the interaction between purified mucopolysaccharides and metachromatic dyes *in vitro* to the conditions met with in tissue sections? As we know little or nothing about the conditions of the acid mucopolysaccharides in their normal environments this question cannot be adequately answered. Neverthe-

less, it seems to be justified to make a few suggestions concerning the validity of the metachromasia found in connective tissue sections. *The specificity of the metachromatic staining, when performed with the precautions outlined by Lison, is fairly high.* Within adequately prepared sections of loose connective tissue extracellular metachromasia indicates the presence of acid mucopolysaccharides with or without sulfuric acid groups. On the other hand, the absence of metachromasia must not be interpreted as being dependent on the absence of mucopolysaccharides. Bank and Bungenberg de Jong (1939) explained metachromasia as a process of electro-absorption of dye cations to the negative charges of a highly polymerized biocolloid. Within chondroitin sulfuric acid and hyaluronic acid the sulfate groups and the carboxyl groups are the active groups and must be freely accessible to the dye cations and must furthermore possess a certain density to produce a significant metachromatic reaction. As pointed out by Sylvén and Malmgren, formation of complexes of mucopolysaccharides and other substances like protein may destroy the metachromatic property of the mucopolysaccharide. With regard to the hyaluronic acid this may be non-metachromatic even if existing in a free form, depending on a low actual degree of polymerization.

*Summary. — Purified sulfur-free hyaluronic acid may exhibit a significant metachromatic potency when tested in vitro with purified toluidine blue. Different preparations show a pronounced variability in their metachromatic potency. The chromotropic activity of a certain preparation of hyaluronic acid is obviously related to the actual physicochemical state of the substance concerned.*

The lowest dry weight concentration of the hyaluronic acid preparations tested producing a distinctly visible metachromasia was approximately 0.5 %.

The effect of purified testicular hyaluronidase on the metachromatic potency of hyaluronic acid varied significantly when tested on different preparations.

## Reaction of purified acid mucopolysaccharides with the Schiff leucofuchsin reagent after oxidation with periodic acid and sodium bismuthate

The primary reason for these investigations was an attempt to elucidate the correlation, if any, between metachromatic potency and PAS-reactivity of various preparations of hyaluronic acid. In these studies most of the hyaluronic acid samples showed a surprisingly low reactivity with Schiff's leucofuchsin after treatment with periodic acid when used

as prescribed by Hotchkiss (1948). This finding led to the more systematic investigations reported in this part of the work.

Hotchkiss characterizes hyaluronic acid as yielding a very strong reaction with Schiff's reagent after oxidation with periodic acid. No data are given on the hyaluronic acid preparation used in his experiments. Moreover, he based his statement on the results gained with the use of "spot tests" which are not comparable to ordinary histological technics.

Furthermore, the PAS-reaction is often of a low intensity in tissue structures known to contain high amounts of hyaluronic acid, whereas a rather strong reaction may be found in structures which are said to be free from hyaluronic acid (cartilage) but rich in the PAS-negative chondroitin sulfuric acid. It seemed desirable, therefore, to test this substance as well as heparin with respect to their PAS-reactivity by using a technic comparable to that commonly employed on histological sections.

## Material and methods

Specimens of the same material as employed for the studies on metachromasia were used in these investigations. No test tube experiments were however performed.

Dried and fixed model smears of the type described above were used. Principally three different types of model smears were employed. One contained hyaluronic acid (from umbilical cord, Sample H 62; see Table 3 page 32), chondroitin sulfuric acid (from beef nasal septum), heparin (commercial source), and crude liver glycogen. The second type of model smear contained the three mucopolysaccharides mixed with serum (ratio 1 : 2) plus serum alone. In the third type of model smear three different hyaluronic acids were applicated in the gelatine film (Samples H 33, H 58 and H 66; see Table 3, page 32). In addition dried and fixed smears of vitreous body and synovial fluid were used.

*Reagents.* — Schiff's leucofuchsin was prepared according to the description made by Hotchkiss (1948). Three different batches of basic fuchsin were tested with respect to the amount of formaldehyde required to recolor a certain amount of leucofuchsin. No significant differences were found between the dye batches at hand. Thus one of the batches was arbitrarily selected for use in the present experiments (Basic fuchsin, Coleman and Bell; Certified dye). When ready for use the leucofuchsin reagent was always perfectly colorless.

*Periodic acid solutions.* — The composition of the oxidizing solution was varied as to pH and alcohol content, as follows:

1. M/5 periodic acid—$H_5IO_6$ (F. Smith Chemicals, Ohio, U. S. A.) — dissolved in M/20 sodium acetate containing 70 % ethanol, pH 1.4 *(Periodic acid 1)*.
2. The same as (1) but without alcohol; pH 1.4 *(Periodic acid 2)*.
3. M/5 periodic acid—$H_5IO_6$—dissolved in M/10 $NaHCO_3$ containing 70 % ethanol; pH 4.0 *(Periodic acid 3)*.
4. The same as (3) but without alcohol *(Periodic acid 4)*.
5. M/7 sodium metaperiodate—$Na_3IO_5$—dissolved in N/10 $HNO_3$; pH 1.8 *(Periodic acid 5)*.

All the oxidizing solutions were used within a few hours after being prepared.

*Sodium bismuthate.* — M/20 sodium bismuthate—$NaBiO_3$ (B.D.H.) dissolved in M $H_3PO_4$—orthophosphoric acid. This solution was used immediately after preparation.

*Reducing solution.* — This solution was prepared as prescribed by Hotchkiss: potassium iodide, 1 g, and sodium thiosulfate, 1 g, dissolved in 50 ml N/50 HCl containing 70 % ethanol.

*Sulfite water.* — Potassium metabisulfite, 0.4 g, was dissolved in 100 ml N/10 HCl.

*Procedures.* — The staining procedure principally followed the scheme outlined by Hotchkiss (1948); oxidation in periodic acid followed by reduction of the periodic acid residue, staining in leucofuchsin solution, treatment with sulfite water to prevent nonspecific reactions with leucofuchsin, and, after rinsing in water, dehydration and mounting.

In the present series of experiments the procedure outlined was varied with respect to the oxidation procedure according to the data given in Table 5. Furthermore the influence of the reducing rinse devised by Hotchkiss was examined in some specimens.

In a series of experiments oxidation was performed with sodium bismuthate in place of periodic acid. According to Rigby (1949, 1950) this substance acts in a similar way as periodic acid by cleavage of 1.2-glycol linkages with the production of aldehydes. The bismuth pentoxide formed at the reaction adheres to the smears in the form of brown crystals which, however, are removed by a brief rinse in N HCl (in 70 % ethanol) followed by washing in 70 % ethanol. No reducing agent is employed after the oxidation but the other steps of the procedure are identical with those in Hotchkiss' method.

After the oxidation procedure all the model smears were stained in leucofuchsin for 30 minutes and washed in three changes of sulfite water for 2 minutes each. Followed dehydration in ethanol and mounting in balsam.

Control slides were not oxidized but were carried through the other steps of the procedure.

Where a negative result was obtained, the presence of the substrate in the gelatine film was demonstrated by subsequent staining in 0.1 % toluidine blue stain.

The effect of testicular hyaluronidase (Hyalas, Leo; 10 V.R.U./ml citrate buffer pH 6.2) on the staining results was tested on smears of hyaluronic acid-serum mixture. The time of incubation was 4 hours at 40° C. Control slides were incubated under the same conditions in buffer solution only.

## Results

The reactions observed are summarized in Table 5. As shown in this table the hyaluronic acid preparations reacted weakly, if at all, with the Schiff reagent even after comparatively extensive treatments with the various oxidating solutions. A certain difference in reactivity between the hyaluronic acid preparations was noted. Thus, sample H 58 (from vitreous humor; low relative viscosity) and H 33 (from umbilical cord; comparatively low relative viscosity) seemed to possess more reactive groups than samples H 66 and H 62 (highly viscous preparations from umbilical cord; see Table 3).

The oxidizing power of periodic acid seems to be somewhat depressed in alcoholic solution (Periodic acid 1), as compared to an aqueous solution of the agent (Periodic acids 2 and 5).

The reducing rinse apparently interferes with the coloring of the aldehydes produced by the oxidizing agents. This effect is, however, significant only when the reactivity of the substrate towards leucofuchsin is low. Concerning glycogen, for example, the brilliancy of the stain of this substance is completely unaffected by treatment with the reducing solution.

As seen from the table, chondroitin sulfuric acid as well as heparin gave significant reactions when the hyaluronic acids were completely uncolored (see Fig. 7: 3).

When mixed with serum all the mucopolysaccharides gave strong reactions. (Fig. 7: 6.) Serum alone produced a significant reaction even when oxidized for a short time. (Fig. 7: 5.) The smear of synovial fluid showed a strong reaction whereas vitreous humor took a rather faint color.

The vigorous reactions given by glycogen is an outstanding finding in all the experiments performed. (Fig. 7: 1—3.)

The use of aqueous solutions of periodic acid is limited with respect to the solubility of the mucopolysaccharides. As shown in the table, parts of the substrates were dissolved in the aqueous oxidizing solution when the treatment exceeded 15 minutes.

Where negative results were obtained it was almost always possible

Table 5. *Responses of Acid Mucopolysaccharides to the Schiff Reagent after Periodic Acid or Sodium Bismuthate Oxidation at Room Temperature.*

(HyA = hyaluronic acid; ChS = chondroitin sulfuric acid.)

| Substrate | Reducing rinse | Periodic acid 1 | | | Periodic acid 2+5 | | | Periodic acid 3+4 | | Sodium bismuthat | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 15 min. | 60 min. | 180 min. | 5 min. | 15 min. | 30 min. | 15 min. | 60 min. | 5 min. | 15 min |
| HyA — H 62 ..... | + | o | (+) | + | o | (+) | (+) | o | o | – | – |
| " ..... | – | o | + | + | o | + | (+) | o | o | o | (+) |
| HyA – H 62 + serum | + | + + | + + | + + | + | + + | + + | + | + | + | + |
| HyA — H 66 ..... | + | o | o | (+) | o | (+) | (+) | o | o | o | (+) |
| " ..... | – | o | (+) | + | o | + | (+) | o | o | – | – |
| HyA — H 58 ..... | + | (+) | + | + | o | + | o* | o | + | o | + |
| HyA — H 33 ..... | + | o | (+) | + | o | + | o* | o | (+) | o | + |
| ChS — NI ..... | + | (+) | + | + + | (+) | + | (+)* | (+) | + | + | + + |
| ChS — NI + serum. | + | + + | + + | + + | + | + + | + + | + | + | + + | + + |
| Heparin ..... | + | + | + + | + + | + | + + | + * | + | + | + | + + |
| Heparin + serum ... | + | + + | + + | + + | + | + + | + + | + | + | + | + + |
| Glycogen ..... | + | + + + | + + + + | + + + + | + + | + + + | + + * | + + | + + | + + + | + + |
| Serum ..... | + | + | + + | + + | + | + + | + + | + | + | + | + + |
| Synovial fluid (fixed) | + | + + | + + | + + | + | + + | + + | + | + | + | + + |
| Vitreous humor (fixed) ..... | – | + | + | + | + | + | (+)* | (+) | + | (+) | + |

* Partly dissolved during the treatment.

to demonstrate the presence of the test substances in the gelatine films with the aid of toluidine blue staining (see Fig. 7: 4 and 8).

The controls were completely unstained in all cases.

The effect of hyaluronidase on the staining reactions seemed to be rather vague. *When the hyaluronic acid-serum mixtures or the smears of native tissues were treated with the enzyme, the subsequent PAS-staining seemed to be more brilliant than that observed on the controls.*

The use of sodium bismuthate as oxidizing agent did not improve the reaction of hyaluronic acid with the Schiff reagent.

*Discussion.* — The vigorous reaction of isolated hyaluronic acid with the Schiff reagent after periodic acid oxidation as reported by Hotchkiss (1948), Jorpes *et al.* (1948) and others could not be observed in the present studies. As pointed out above, the "spot test" technic used by Hotchkiss is not comparable to the ordinary histological technic. In the spot tests the surplus of the reagents as well as all the soluble products of the oxidation are retained in the spot whereas these substances are removed by washing and rinsing of the histological sections. The technic used in the present investigations makes it possible to draw comparisons with the reactions found in ordinary tissue sections.

Obviously, purified preparations of hyaluronic acid may react with

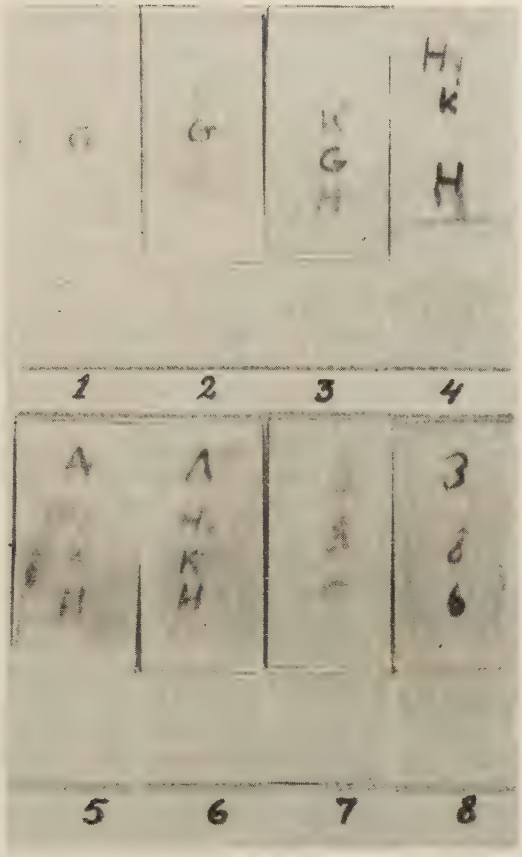


Fig. 7. Fixed smears of various plysaccharides stained with the PAS reaction and toluidine blue (4.8). For details see the text.

(A = serum; G = glycogen; H = heparin; Hy = hyaluronic acid — H 62; K = chondroitin sulfuric acid; 3 = H 33; 6 = H 66; 8 = H 58; see Table 3, p. 32.)

leucofuchsin when the oxidation with periodic acid is allowed to continue long enough. (See Table 5 and Fig. 7: 7.) But even then the color obtained with the Schiff reagent is comparatively faint.

In the present experiments the hyaluronic acid preparation which seemed to be *the most depolymerized one (H 58) produced the strongest PAS-reaction, whereas the highly viscous samples H 62 and H 66 were practically PAS-negative even after 3 hours' oxidation* (Fig. 7: 3 and 7).

After the present work was finished two reports have appeared on the stainability of purified mucopolysaccharides with the PAS method (Davies, 1952; Glegg *et al.*, 1952). The technics used in these works

are similar to those described here. Davies found a pure, sulfur-free hyaluronic acid preparation to give no reaction whereas a crude, commercial preparation, as well as an undegraded hyaluronic acid complex from ox synovial fluid, both reacted vigorously with the PAS-reagents. Glegg *et al.* reported three different samples of pure hyaluronic acid to give negative results. It must be noted that the workers cited used the PAS-technics described by Hotchkiss (1948) and McManus (1946), respectively. No trials with extended periods of oxidation were made.

As shown in Table 5 the influence of the variations in pH on the oxidizing power of the periodic acid solution was negligible at room temperature.

The faint or negative reaction of purified hyaluronic acid with the PAS-reagents is in agreement with the findings of Meyer and Fellig (1950), Jeanloz (1950), and Blix (1951) that hyaluronic acid consumes small amounts of periodic acid (about 1 mole per 10 disaccharide units). Jeanloz suggests that the glucosidic linkages in hyaluronic acid are of 1,3-type. Thus the reactive glycol groups would be blocked by this linkage. According to Meyer and Fellig the periodic acid attacks only the end groups of the long chain structure, leaving the interior of the molecule intact. Blix, however, points out that the consumption of periodic acid is too large to be explained only by an attack of the end groups of a linear long chain structure. He suggests that hyaluronic acid may possess alternating 1,3 and 1,4-linkages or an irregular structure the type of which may vary in hyaluronic acids of different origin. From a histochemical point of view this is of a certain interest. It justifies the assumption that *it is inadequate to interpret a reaction found with a certain hyaluronic acid preparation in vitro or within a special tissue area as implicating hyaluronic acids in general.*

As seen from Table 5 and in Fig. 7: 3 (K) the chondroitin sulfuric acid preparation used (from beef nasal septum) gave a significant reaction with the Schiff reagent when treated for a sufficiently long period with periodic acid. This finding is somewhat confusing as the chemists have reported that this mucopolysaccharide consumes insignificant amounts of periodic acid (Meyer *at al.* 1946, 1948; Jorpes *et al.*, 1948; Blix, 1951). Recently, however, Wolfrom *et al.* (1952) found a large consumption of periodic acid when the oxidation of ChS was prolonged.

As pointed out above, tissue structures rich in ChS like cartilage often give a comparatively strong PAS-reaction. Glegg *et al.* (1952) suggest that the positive reaction of tracheal cartilage depends on some other polysaccharide than ChS. Davies (1952) who found a sodium salt of ChS to give a vigorous reaction with the Schiff reagent after a comparatively short oxidation with periodic acid, does not enter into any speculations concerning the bearing of his observations.

The positive reaction given by the commercial heparin used in the present studies may be explained by its content of monosulfated heparin

which is known to consume significant amounts of periodic acid (Jorpes *et al.*, 1948).

The relatively strong color given by the mucopolysaccharide-serum mixtures seems mainly to be due to the reactivity of the serum component. As shown by Hotchkiss (1948) and Glegg *et al.* (1952) muco-proteins and glycoproteins of serum give a positive PAS-reaction.

The enhancing effect of testicular hyaluronidase on the coloration of hyaluronic acid with the Schiff reagent may be assumed to be due to liberation of reactive units by a cleavage of the polysaccharide molecule.

Histochemical inferences from the observations made must be drawn with several reservations. It may be justified, however, to assume that certain *highly polymerized hyaluronic acids are hardly demonstrable with the PAS-reaction. Depolymerization seems to increase the reactivity of these hyaluronic acids towards the PAS reagents.*

*Part of the color found in connective tissue after PAS-staining might be due to the presence of chondroitin sulfuric acid or heparin derivatives.*

*Summary.* — Five different samples of hyaluronic acid from various sources applied as smears on glass slides gave faint or negative reactions with Schiff's leucofuchsin reagent after oxidation with periodic acid or sodium bismuthate. After prolonged oxidation in periodic acid some of the hyaluronic acid samples showed a faint reaction. Chondroitin sulfuric acid as well as heparin gave positive reactions under conditions where a hyaluronic acid sample was completely unstained.

Mixtures of serum and mucopolysaccharides, as well as fixed smears of synovial fluid, gave strong reactions with the Schiff reagent even after moderate oxidation in periodic acid. Vitreous body smear showed a rather faint reaction.

*Treatment with testicular hyaluronidase for a period long enough to abolish most of the metachromatic properties of the substrates seemed to increase their reactivity towards the PAS reagents.*

## Other histochemical methods for demonstration of acid mucopolysaccharides

*Hale's technic for acid mucopolysaccharides.* — This method is based upon the adsorption of colloidal ferric hydroxide on acidic tissue components. The iron adsorbed is demonstrated by the Prussian blue reaction with potassium ferrocyanide. According to Pearse (1951), Davies (1952) and Gomori (1952) the technic is not specific to acid mucopolysaccharides. Hale (1946) stated that neutral mucopolysaccharides

Table 6. *Reactivity of Acid Mucopolysaccharides towards Hale's Colloidal Iron, Alone and in Combination with Periodic Acid-Schiff Staining.*

(HyA = hyaluronic acid; ChS = chondroitin sulfuric acid; Hyas = hyaluronidase).

| Substrate | Hale's colloidal iron | Hale's colloidal iron + PAS |
|---|---|---|
| HyA — H 62 ..... | bluish green | dark green |
| HyA — H 62 + serum.. | bluish green | dark green |
| H 62 + serum + Hyas... | green | green |
| HyA — H 58 ..... | dark bluish green | gren + pink |
| ChS — NI ..... | dark bluish green | bluish green |
| Heparin (commercial).. | light green | pink + light green |
| Glycogen..... | no reaction | deep violet |
| Vitreous body (fixed) .. | green – bluish green | green with streaks of violet fibrils |

as well as proteins do not combine with the dialyzed ferric hydroxide. Grishman (1948), in her studies of the mucopolysaccharides in normal tissues, found that only those mucins which stain metachromatically showed a distinct Hale reaction. On the other hand, she found metachromatic mucins which did not react with the Hale reagents.

*Own observations.* — Model smears similar to those described above (see page 33) were stained according to the method devised by Hale (1946). It was found that all the hyaluronic acid preparations took the colloidal iron stain. When rendered non-metachromatic by treatment with testicular hyaluronidase (Hyalas, Leo — 10 V.R.U./ml citrate buffer, pH 6.2), the smears still showed a bluish green or green color with the Hale reagents (see Table 6).

Chondroitin sulfuric acid showed a strong reaction, whereas the commercial heparin product took the iron stain to a slight degree only. The crude liver glycogen preparation was entirely negative.

Dried and fixed smears of vitreous humor and synovial fluid gave a strong bluish green color which was insignificantly weakened by a 4-hours treatment with testicular hyaluronidase. However, such a treatment more or less completely abolished the chromotropic activity of the smears.

*Comment.* — The Hale reaction cannot be considered specific to any of the acid mucopolysaccharides. *A depolymerized specimen of hyaluronic acid or its complex with protein(s), showing no metachromatic potency, may be rendered visible by the Hale method.*

*Staining with a combined Hale-PAS-technic.* — Ritter and Oleson (1950) were the first to suggest the Hotchkiss stain as a counter-stain to Hale's reaction. Each stain, when applied by itself to tissue sections, is practically identical with the other in distribution. Yet these authors stated that in combination the two stains are seen side by side in parallel locations and can be recognized as two distinct chemical reactions. The in-

terpretation of the different reactivity observed does not seem to be clear. The authors performed no studies with that method on purified mucopolysaccharides *in vitro*. A somewhat modified combined staining with colloidal iron and the PAS reagents has been described by Rinehart and Abul-Haj (1951). The staining results gained with this method are very incompletely described by the authors.

*Own observations.* — Model smears similar to those used in the studies reported above were employed. The effect of testicular hyaluronidase on the staining results was studied on smears incubated with the enzyme for 4 hours at 40° C (the enzyme solution was composed as described above). The following procedure was employed in all experiments:

After staining in Hale's reagents as devised in the original description, the smears were oxidized in alcoholic periodic acid (Periodic acid 1 ; see page 44) for 60 minutes at room temperature. After that the slides were rinsed in the reducing solution of Hotchkiss and were then treated with Schiff's reagent for 45 minutes. After washing in sulfite water the smears were dehydrated in alcohol and mounted in balsam.

Control smears were taken to the Schiff reagent directly after the treatment in Hale's reagents, thus omitting the oxidation step.

The observations are summarized in Table 6 where the staining results obtained with the ordinary Hale technic are also indicated.

As seen from the table, most of the acid mucopolysaccharides retained their Prussian blue color. The highly depolymerized hyaluronic acid preparation H 58 as well as heparin showed a double coloration. In the microscope the hyaluronic acid concerned showed a core of greenish precipitates surrounded by a pink, homogeneously stained area. The heparin smear was macroscopically pink, but in the microscope small greenish precipitates were observed here and there within the pink substance. As could be expected, glycogen took a strong violet color with the Schiff reagent.

In all the cases where the oxidation step was omitted no traces of Schiff reactive material could be found.

*Comment. — Most of the hyaluronic acid preparations were more reactive towards the colloidal iron than towards the PAS reagents.* When combining the two staining methods as in the present studies, most of the acid mucopolysaccharides retained the Prussian blue color. The hyaluronic acid which showed a double coloration was the one that took a significant color with the PAS-method after one hour's oxidation (see Table 5). Likewise heparin showed a significant PAS-reaction under the same conditions.

It may be that the same substrate prefers one or the other of the reagents depending on the actual degree of polymerization and, consequently, of the configuration of the aggregates of molecules. Yet too little is known of the chemistry of the mucopolysaccharides to justify

much speculation in this direction. In any case, the present results further confirm the suggestion that *different preparations of an acid mucopolysaccharide may behave histochemically like entirely different substances.*

## Conclusions

The acid mucopolysaccharides of the ground substance cannot under all conditions be histochemically indicated or identified with the use of one single staining method. The reactivity of these substances towards the methods at present available for their histological demonstration seems to vary considerably. The observations made in the present study are a contribution in support of the assumption that the mucopolysaccharides in general, and hyaluronic acid in particular, possess a certain degree of plasticity with respect to the physico-chemical constitution. The actual supply of accessible reactive groups like carboxylic, sulfate and 1,2-glycol groups seems to be the factor which ascertains in the main the staining results obtainable with the histochemical methods studied in this work.

At present no histochemical technics are available which may be considered *a priori* to be specific to any particular constituent of the connective tissue ground substance. The periodic acid-Schiff reaction and Hale's stain are more or less unspecific reactions.

Though obscure as to its physico-chemical nature, *metachromasia* is to be considered the most useful indicator of the presence of acid mucopolysaccharides within connective tissues. When produced under certain strictly standardized conditions, this reaction possesses a high degree of specificity. This is, however, related to the *physical conditions* of the substrate rather than to its chemical composition. Purified acid mucopolysaccharides isolated from connective tissue all possess chromotropic potency *in vitro*. The chromotropic activity of the acid mucopolysaccharides varies with their actual physico-chemical state. This particularly applies to hyaluronic acid, the metachromatic potency of which fluctuates significantly with different preparations. *Hyaluronic acid produces a significant metachromasia only when highly polymerized.* Furthermore, it may be assumed that differences in the structure of the mucopolysaccharide may affect its chromotropic potency. *It seems justified to presume that under certain conditions hyaluronic acid significantly contributes to the metachromasia obtained within loose connective tissue specimens.*

Metachromasia in general and that produced by hyaluronic acid in particular is highly dependent on the quality of the dye used.

The value of the *periodic acid-Schiff reaction* as a tool for demon-

stration of purified acid mucopolysaccharides is limited. Purified hyaluronic acid may be completely unstained even after prolonged treatment with the oxidant. *When sufficiently depolymerized, hyaluronic acid may show a significant positive reaction with the periodic acid-Schiff reagents.* The "native" hyaluronic acid-protein complex as found in synovial fluid gives a positive periodic acid-Schiff reaction. The mucin clot formed when hyaluronic acid is mixed with acidified serum likewise shows a strong positive Schiff-reaction. Serum itself is stained quite intensely with this method.

Chondroitin sulfuric acid and commercial heparin are stainable with the periodic acid-Schiff method *in vitro.*

Hyaluronic acid, chondroitin sulfuric acid and heparin show, *in vitro,* a definite *affinity to colloidal iron.* Hyaluronic acid takes a heavy color with the Hale method even when the polysaccharide is strongly depolymerized. When combining the periodic acid-Schiff method with the Hale technic, comparatively high polymerized specimens of hyaluronic acid retain the Prussian blue whereas depolymerized hyaluronic acid may be partly stained by the Schiff reagent.

It may be concluded that highly polymerized hyaluronic acid may be stained metachromatically with satisfactory thiazin dyes or with Hale's iron reagent. The depolymerized mucopolysaccharide possesses no metachromatic activity but may react with Schiff's reagent after an adequate treatment with periodic acid. In addition, it retains the affinity to iron even when highly depolymerized and may thus be visualized with Hale's technic.

Chondroitin sulfuric acid is demonstrable with the metachromatic reaction, with Hale's method or, to a certain degree, with the periodic acid-Schiff reagents. This statement might be valid only for the chondroitin sulfuric acid present in cartilage.

# II

# Organization of the Ground Substance in Ascorbic Acid Deficiency and its Modification by the Action of Cortisone

CHAPTER IV

# Introductory remarks on the function of the ground substance

Though most histologists are in agreement about the morphological structure of loose connective tissue, opinions diverge concerning the functions of this tissue and especially of its ground substance. This seems to be due to lack of conclusive data on the organization of the matrix in the normal state and on the formation and turnover of its various constituents. Yet there are some concepts regarding the physiology of the matrix which are commonly accepted and which will be shortly mentioned below.

The notion that loose connective tissue functions as an inert supporting stuff only, must nowadays be rejected in view of the evidence gained on its reactivity to various physiological stimuli and its prominent sensitivity to direct physical and chemical intervention. Disorder of the function of the tissue manifests itself as changes of the structure or function of the extracellular elements. Reactive processes can, however, hardly be expected to be initiated by these elements but rather by the cells. These are believed to regulate the structural and physicochemical organization of the extracellular components, probably by some kind of secretory activity.

The functional condition of the matrix is determined in part by the mucin it contains. The preservation of normal tissue dynamics presumably depends on an adequately organized ground substance gel. This seems to be very plastic, its form shifting between the fluid-like and the more or less rigid gel-like. These conditions are attributed to the content of the highly molecular acid mucopolysaccharides hyaluronic acid and chondroitin sulfuric acid.

The fundamental functions attributed to the ground substance are all related to and affected by the degree of polymerization and/or aggregation of the constituents of the matrix. Its acting as a medium for transport and for storage of water and electrolytes as well as its shock-absorbing and lubricating effect depend upon the plasticity of its state of polymerization. This is also of significant importance for the "barrier-effect" exerted by the ground substance against penetration by foreign matter into the tissue. Significant variations in the

resistance of this barrier under physiological as well as pathological conditions have been reported in the literature.

One function recently introduced into the discussion of these problems is the possible role of the sulfated mucopolysaccharides as cationic exchange resins. The importance of such a function to the metabolism of the organism is evident. Experimental data on this problem are lacking at present.

The role played by the ground substance in processes of repairs following trauma or inflammation is being intensely studied, but our knowledge of this important question is still defective. Experimental data hitherto accumulated point to the ground substance as an essential factor in the formation of the fibrillar elements.

The permeability of connective tissue structures to poorly diffusible dyes or other colored matter introduced into the tissue is frequently used as indicator of the actual state of the ground substance gel. By using this technic considerable knowledge has been gained concerning factors that may modify the structural organization of the matrix. In this way the functional system hyaluronic acid-hyaluronidase came into focus. The balance of this system within the connective tissues of the animal organism has become an important physiological problem which has kept a great number of scientists busy during the last decade. (The reader is referred to the reviews made by Duran-Reynals, 1942; Meyer, 1947; Gibian, 1951; and Ragan, 1952. The present brief survey is based on information gained from these reviews.) The existence in the tissues of several physiological factors which inhibit the activity of hyaluronidases further complicates the problem. The question is, however, whether or not the functional system mentioned is dynamically operative as a regulator of the physico-chemical organization of the ground substance.

According to experimental data accumulated, there is good reason to assume that the condition of the ground substance is influenced by hormones as well as by vitamins. The dramatic effect of castration or administration of estrogens on the structure of the cock's comb is a well-known example of hormonal influence on loose connective tissue. As another example the changes of the ground substance of dermal connective tissue in myxedema may be mentioned. The striking effect of certain corticosteroids on connective tissue diseases reported by the clinics has stimulated the experimental study of the effect of these hormones under normal conditions. Thus it has been shown that cortisone has en inhibiting effect on the formation of granulation tissue in wounds. Furthermore this hormone as well as ACTH seem to modify the permeability of the dermal connective tissue.

It has been pointed out that some of the effects of cortisone on connective tissue are similar to those produced by ascorbic acid deficiency.

Ascorbic acid evidently plays some important role in the production of the ground substance constituents. In scurvy very little, if any, matrix is said to be found in regenerating connective tissue.

To sum up, loose connective tissue must be considered a dynamic tissue, presumably active in many functional processes of vital importance to the organism. The preservation of normal tissue dynamics presumably depends upon an adequate organization of the mucopolysaccharide-protein complex constituting the matrix. The mucolytic enzymes might be physiologically operative factors in the mechanism of adaptation of the ground substance gel to various functional conditions.

Knowledge of the formation, and of the turnover rate of the constituents of the matrix, is meager.

Certain hormones and vitamins, primarily the adrenocorticoids and vitamin C, evidently act upon the formation and maintenance of the constituents of the ground substance complex.

## State of loose connective tissue in ascorbic acid deficiency

Ascorbic acid deficiency constitutes an excellent example of the dynamic state of the connective tissue and its reliance upon certain conditions for its adequate preservation (Gross, 1952). In the absence of vitamin C the structural organization of the tissue changes and the morphology resembles that of embryonal mesenchyme (Wolbach, 1933). The effect of experimental vitamin C depletion is restricted to tissues of mesenchymal origin and is simply characterized as a failure in the formation and maintenance of intercellular materials (Höjer, 1924; Wolbach and Howe, 1926; Wolbach, 1933).

Höjer was the first to elucidate the interrelationship between formation of collagen and vitamin C metabolism. He stated that "there is a general atrophy in scurvy of the connective tissue, and especially of its collagen substance". Bourne (1942) reports that native collagen may retrogress in a scar if scurvy is supervened on a recently healed wound. Quantitative studies on the collagen content of vitamin-C-depleted guinea pigs made by Elster (1950) and Robertson (1950) have shown, however, that the concentration of mature collagen does not diminish in either acute or chronic scurvy. On the basis of these findings Elster states that ascorbic acid is essential for the formation of collagen, but once formed, the collagen fibers seem to be independent of further vitamin C nutrition; and he concludes: "in the true sense, therefore, ascorbic acid may not be essential for the maintenance of preformed connective tissue." It may be added that Bourne (1942) found that reticulum may be formed in

scorbutic animals but without maturing into collagen. (For further details on this special subject the reader is referred to the review made by Wolbach and Bessey, 1942, and by Ragan, 1952.)

Regarding the production and condition of the amorphous ground substance of loose connective tissue, original reports are more scarce. Wolbach (1933) states that processes of repair in vitamin-C-deficient guinea pigs have shown that in the state of absolute scorbutus the fibroblasts migrating into the blood clot of a muscular wound produce a liquid material. After administration of vitamin C (orange juice) a homogeneous material resembling amyloid appeared around the cells. This substance was suggested by Wolbach to be "homogeneous collagen". Hunt (1941) describes the formation of an amorphous matrix in granulation tissue of guinea pigs suffering subtotal scurvy. This matrix is traversed by argyrophilic fibrils which, however, retained their argyrophilia without maturing into collagen. These findings have been confirmed by Danielli *et al.* (1945) in studies on guinea pigs on a low vitamin C level. More recently Penney and Balfour (1949) reported the absence of extracellular metachromatic staining substances in wounds of scorbutic guinea pigs, an inhibited formation of fibrils and a limited capillary proliferation. After the intramuscular administration of ascorbic acid (one single dose of 25 mg) there was evidence of increased production of metachromatic staining material after 6 hours, and after 12 hours a large amount of such material was found in the granulation tissue. A correlation was noted between the appearence of the extracellular metachromasia and formation of fibres. On the basis of a treatment of the sections with hyaluronidase and ribonuclease the authors suggest the possibility of hyaluronic acid being responsible for the extracellular metachromasia and ribo-nucleoproteins for the intracellular variety.

Regarding the appearance of the fibroblasts within the granulation tissue in scorbutic guinea pigs the observations reported show a striking conformity. The cells migrate widely and multiply rapidly and seem to have lost their property of differentiation. Their cytoplasm is vacuolated and shows a strong metachromasia. It has been stated that the morphologic effects of ascorbic acid deficiency must be regarded as the result of retarded or suppressed normal activities of cells (Wolbach and Bessey, 1942) which result in quantitative as well as qualitative changes of the intercellular material. It may be added that such a condition is not specific to ascorbic acid deficiency.

Concerning the condition of the ground substance of non-injured connective tissue in vitamin-C-depleted animals, little information is available in the literature. Gersh and Catchpole (1949) in a histochemical study of skin and fascia of three scourbutic, "severely affected" guinea pigs, report that an increased amount of "water-soluble glycoprotein" was found in the tissues mentioned. They suggest that the

ground substance in scurvy is in a depolymerized state. In this connection a theory may be mentioned which was advanced several years ago by Wolbach and Howe (1926) claiming that in scorbutus there is lacking an agent, common to all connective tissues, which is responsible for the jelling of the intercellular substance which would otherwise remain liquid.

From the literature it may be concluded that most histologists agree with respect to the more or less complete absence of amorphous matrix in regenerating connective tissue of vitamin-C-depleted animals. The question whether this is due to an inability of the tissues to form any ground substance or whether an abnormally composed substance, lacking ordinary staining qualities, is produced does not seem to have been discussed as a problem of importance. Based on the absence of metachromatic material in regenerating tissues Meyer (1944) states that the tissues are unable to form chondroitin sulfuric acid in scurvy, and Penney and Balfour (1949) assume that in scurvy there is also an inability of the tissues to produce hyaluronic acid. The report given by Gersh and Catchpole, on the other hand, would seem to justify further investigations of this problem.

Recently a scurvy-like effect on granulation tissue has been experimentally produced by administration of cortisone (Ragan *et al.*, 1949). This finding initiated several investigations of the problem concerning the interrelationship of adrenocortical hormones and vitamin C in the genesis of connective tissue lesions (Schaffenburg *et al.*, 1950; Plotz *et al.*, 1950; Spain *et al.*, 1950; Hyman *et al.*, 1950; Upton and Coon, 1951 and others). A certain interest in the relation of ascorbic acid deficiency to the adrenals developed earlier from the familiar facts that the adrenals contain large amounts of ascorbic acid and that the vitamin is directly implicated in the activity of the adrenal cortex (Giroud, 1940). Giroud and Ratsimamanga (1940) have furthermore postulated that most of the symptoms of experimental scurvy are not due to the ascorbic acid deficiency as such, but are secondary to adrenal insufficiency. These authors could also demonstrate a beneficial effect of cortical extract on some of the conditions of scurvy. This has been confirmed by Schaffenburg *et al.* (1950) using cortisone and by Hyman *et al.* (1950) using cortisone and ACTH. Other workers, on the other hand, did not find any effect of cortisone and ACTH either on general symptoms of scurvy such as fall in body weight (Clayton and Prunty, 1951) or on wound healing in scorbutic guinea pigs (Upton and Coon, 1951, Bourne, 1952). It may be mentioned here that a retarding effect of cortisone on wound healing of normal subjects was not demonstrable in all the investigations performed on this problem. The divergences one finds in the literature may be at question of dosage levels and of species differences. Of special interest in this connection are the studies made by Magarey and Gough (1952) on fibroplastic processes produced

in mice, rats and guinea pigs. Fibroplasia was delayed by cortisone in mice and rats but was not effected in guinea pigs.

Of a certain interest in this connection are the reports made by Layton (1951). He states that cortisone inhibits the incorporation of labeled inorganic sulfate, administered to experimental animals or to tissues in vitro, into the chondroitin sulfate of the connective tissue ground substance. Thus he suggests that the action of cortisone on connective tissues is due to its inhibitory effect upon the synthesis of this mucopolysaccharide.

The action of the adrenal cortex on the hyaluronic acid-hyaluronidase system has been investigated by several authors. Without going into details, a few remarks may be made which may have a bearing upon the observations made in the present work.

Adrenocortical extract as well as cortisone are reported to inhibit the spread of colored indicators injected into dermal connective tissue (Menkin, 1940; Opsahl, 1949; Opsahl *et al.*, 1950). A similar effect has been found after administration of ACTH (Carstensen and Linderholm, 1952) and in conditions with an increased secretion of ACTH (Opsahl, 1949, Linderholm, 1951). On the other hand, adrenalectomy causes a significant increase in the spreading rate (Opsahl, 1949). In an investigation on rats I was able to show that this spread-enhancing effect of adrenalectomy was inhibited by parenteral administration of large doses of ascorbic acid (Persson, 1951).

Adrenal steroids are said to inhibit the action of hyaluronidase (Opsahl, 1949; Seifter *et al.* 1949 a, b; Winter and Flataker, 1950; Shuman and Finestone, 1950; Benditt *et al.*, 1950). It may be mentioned in this connection that ascorbic acid is also reported to have such an effect (Reppert *et al.*, 1951).

In summing up it may be said that experimental data reported in the literature indicate that cortisone (and, perhaps, other glucocorticoids) effects loose connective tissue in a way that resembles that of ascorbic acid deficiency with respect to the delayed formation of fibrils. The principal effect of cortisone is said to be an inhibition of the development of fibroblasts, since once the fibroblast is laid down, collagen and ground substance are deposited (Ragan, 1952). Cortisone is suggested to inhibit the formation of chondroitin sulfuric acid and apparently has a significant influence on the hyaluronic acid-hyaluronidase system of the ground substance.

In an attempt to coordinate the data available on the effects of ascorbic acid deficiency and glucocorticoids on loose connective tissue it must be stressed that the scurvy-like effects of cortisone reported in the literature are mostly found in animals in which scurvy cannot be experimentally produced by stopping the supply of vitamin C. In animals dependent on a sufficient supply of ascorbic acid the "scorbutic effect" of the glucocorticoids is apparently much less pronounced or

not demonstrable at all. The beneficial effect on some general symptoms of scurvy demonstrable in guinea pigs has been explained either in terms of sparing ascorbic acid (Hyman *et al.*, 1950) by decreased utilization of the vitamin or as a protection against the non-specific stress of ascorbic acid deficiency (Upton and Coon, 1951). A third explanation is given by Schaffenburg *et al.* (1950) who advance the theory that ascorbic acid deficiency may lead to an unbalance of the hormonal production in the adrenal cortex with an inhibited release of carbohydrate-active steroids. As it is outside the scope of the present work to enter into the problem of the interrelationship of the adrenocortical function and vitamin C, no further discussion of this problem will be carried on.

## Object of the author's investigation

The statement made by various authors that the production of ground substance is inhibited in experimental scurvy is based on the absence of metachromatically stained, extracellular material in granulation tissue. The propriety of such an interpretation may be open to question with respect to the method used, and the reported increase of "depolymerized glycoproteins" in connective tissues of scorbutic guinea pigs might be demonstrated by the use of other histochemical methods.

It thus seemed justified to make an attempt to penetrate this problem a little further by use of the various histochemical methods discussed in the first part of this work.

The questions to be answered were these: Is there any production of ground substance in the loose connective tissue of scorbutic animals? If so, is this ground substance histochemically and functionally different from that formed under normal conditions?

In order to elucidate these problems, loose connective tissue of normal and vitamin-C-depleted guinea pigs was studied by the histochemical and physiological methods to be described in Chapter V. In addition, part of the material was analyzed chemically with respect to the content of amino sugars in order to obtain some quantitative data on the ground substance present in the various conditions.

The effect of the glucocorticoids on the ground substance in scurvy is obscure. In the present work the effect of adrenalectomy as well as administration of cortisone on the loose connective tissue of scorbutic animals has been studied with the methods mentioned above. The intention was to find out if the effect of scurvy on the production and condition of loose connective ground substance could be in some way modified by physiological stimuli. The choice of cortisone as such a stimulus was made without any intention of going further into the problem of the interrelationship of the adrenocortical hormones and vitamin C with respect to their action on connective tissues.

CHAPTER V

# Experimental animals

Apparently healthy, female guinea pigs were used throughout. Out of a total number of 270, 97 were used in the elaboration of the operative technique and dietary regimen as well as for preliminary tests, the remaining 173 being used as material for the studies reported here.

As the animals came from different breeders they were all caged in our department for at least one week before being put into any experiments.

When taken into experiments the animals weighed about 275—400 g. During the quarantine period they were given hay, oats, carrots and swedes ad libitum and were weighed every other day.

*Dietary regimens.* According to the experimental purposes the animals were divided into three different groups with respect to the diet given.

A. Animals given a special *scorbutogenic diet* (basal diet) supplemented by *10 mg ascorbic acid* a day given per os.
B. Animals given the basal diet plus *0.1 mg ascorbic acid* a day injected i. m.
C. Animals given the *scorbutogenic* diet only.

The animals were given a mixture of 0.4 ml cod liver oil and 0.1 ml tocopherol once a week by oral feeding.

*Composition of the scorbutogenic diet.* — This diet is principally composed according to Kirk and Tressler (1941) containing

| | |
|---|---|
| Oaten grits | 600 |
| Wheat bran | 200 |
| Dried brewer's yeast | 80 |
| Whole milk powder (heated) | 60 |
| NaCl (iodized) | 20 |
| $CaCO_3$ | 20 |

The whole milk powder was heated in an oven at 100° C in shallow pans for 20—24 hours.

The diet was tested with respect to its content of essential food

Table 7. *Grouping of Experimental Animals.*

| Group | No. of animals | Diet | Days on diet | Treatment |
|---|---|---|---|---|
| I | 10 | Basal + 0.1 mg asc. acid/day | 9—14* | Sham operation |
| II | 10 | ,, | 9—12* | Adrenalectomy |
| III a | 24 | Basal diet | 13—16* | Sham operation |
| III b | 20 | ,, | 16—21 | No operation |
| IV | 25 | ,, | 13—21* | Sham oper. + Cortisone 5 mg/100 g/day |
| V | 16 | ,, | 11—22* | Adrenalectomy |
| VI | 6 | ,, | 17—20* | Adrenalectomy + Cortisone 5 mg/100 g/day |
| VII a | 8 | Basal diet + 10 mg asc. acid/day | 9—12* | Sham operation |
| VII b | 20 | ,, | 8—14 | No operation |
| VIII a | 6 | ,, | 12—14* | Sham operation + Cortisone 5 mg/100 g/day |
| VIII b | 6 | ,, | 12—14* | Adrenalectomy + Cortisone 5 mg/100 g/day |
| VIII c | 6 | ,, | 12—14* | Adrenalectomy |
| IX | 16 | Basal diet | 14—16* | Skin wounds — local treatment |

* Before operation.

factors on 30 growing guinea pigs. All the animals placed on the basal diet alone developed typical scurvy within three weeks and they were all dead within five weeks. When the diet was supplemented with sufficient amounts of ascorbic acid (10 mg per animal per day) no signs of retarded growth or symptoms of scurvy were detectable. All the animals were given water *ad lib.*

When put into the experiments the animal were weighed and a sample of blood was taken by heart puncture for determination of the ascorbic acid concentration. After that the animals were placed in cages, five animals in each. Weighing was performed every other day during the experimental period. When body weights began to decline or at operation, the animals were isolated from one another in small cages and kept in a special room at a constant temperature of + 22—+ 23° C until the end of the experiment.

To study the influence of adrenalectomy and cortisone on the scorbutic material, the vitamin-C-depleted animals were divided into four main groups. Two of these comprised animals which were adrenalectomized, the other two groups were sham-operated. Cortisone was administered to one adrenalectomized and one sham-operated group. For comparative histological studies a smaller part of the animals fed on an adequate diet were treated in a similar way as the scorbutic

ones, with respect to operations as well as the administration of cortisone.

The adrenalectomized animals which received no cortisone were given physiological saline to drink.

The grouping of the material is summarized in Table 7.

The animals used for histological studies were killed at the 2nd, 5th and 8th day after operation, 2—6 animals each time. The animals in Groups IIIb and VIIb were killed at the 16th to 21st day and at the 8th to 14th day respectively. Within Group IV 20 guinea pigs were killed at the 6th to 8th day; the remaining five were used for histological studies only.

## General methods

1. *Surgical technique.* — All operations were made under ether narcosis and sterile precautions. The middle part of the back was depilated by the use of a paste containing BaS, ZnO, and wheat starch (10 : 7 : 12). The depilated skin area was thoroughly washed with soap and water, followed by washing with 1 pro mille solution of a detergent (Quatrogan, Recip) in 70 % alcohol. *Adrenalectomy* was carried out by the dorsal route through a medial incision about 2 cm long. The adrenals were carefully detached from the surrounding tissues and were taken out together with the attached part of the renal capsule. As the adrenals of guinea pigs more or less rigidly adhere to the big vessels passing by (the vena cava and the portal vein), the shelling out of the glands was often followed by large bleedings. The operative mortality was thus rather high (about 25 per cent during the operation or within the following few hours).

The muscle layers were closed by catgut sutures and the skin incision either with stainless steel wire or with wound clips. Special attention was paid to an exact closure of this incision.

In connection with the operation 5 000 I. U. of penicillin (Penicillinnatrium, Astra) in 5 ml saline was injected intraperitoneally.

*Sham operation* was carried out exactly like the operation described above but the adrenals were left intact.

*Implantation of plates of elder-pith.* — To study the effect of hyaluronidase on granulation tissue, two small cylindrical plates of sterilized elder-pith were implanted subcutaneously in connection with the operations described above. The plates were sterilized by autoclaving and dried under sterile precautions in vacuum. When completely dry they were plunged either into a solution of hyaluronidase (Hyalas, Leo, containing 25 V.R.U./ml) or into physiological saline. Inbibition was performed *in vacuo* and the plates were stored in the solution until implanted. To obtain information on the approximate amount of hyal-

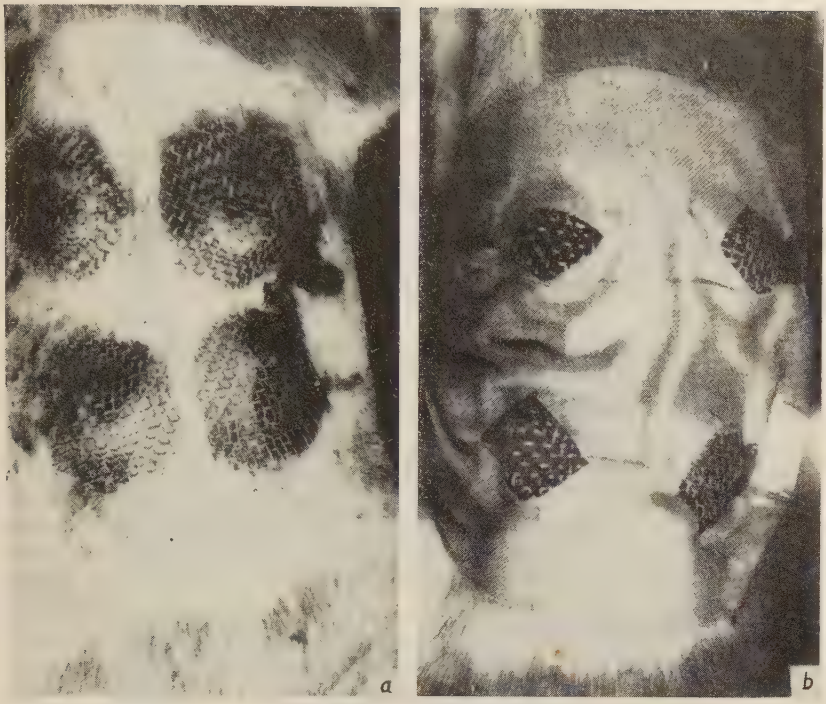


Fig. 8. Circular skin wounds with net-shields undressed (a) and partly covered with plaster (b).

uronidase within the plates after inbibition for a certain time (one hour), a number of the plates were weighed before and after this procedure. The approximate amount of hyaluronidase within a plate of the size given (about 0.1 $cm^3$) was 2—3 V.R.U.

Two elder-pith cylinders were implanted in each animal, one containing hyaluronidase and the other saline. The implantation was performed from the skin incision and the cylinders were placed subcutaneously some distance caudally to the scapulae, symmetrically on either side of the spinal column.

*Circular skin-muscle wounds.* To study the local effect of ascorbic acid, dehydroascorbic acid, and glucuronic acid on the granulation tissue, four circular wounds were produced on each animal within group IX (see Table 7); they were placed symmetrically with respect to the spinal column with two wounds on either side, the anterior ones some distance caudally to the scapulae and the posterior ones in the middle of the trunk (see Fig. 8).

The wounds, 6—8 mm in width, were cut out with curved scissors

so that the skin with the cutis muscle and the superficial part of the subadjacent trunk muscles were removed.

The wounds were covered with convex nets of stainless steel which were fixed to the intact skin outside the wound with two sutures (Fig. 8 a). These "net shields" were further attached to the trunk with a fenestrated strip of plaster. To protect the open wounds from contamination with dirt from the cages, the nets extruding through the holes of the plaster were covered with sterile compresses which were easily removed at the application of the various drugs.

The substances to be tested were applied to the wound area by spraying with an ordinary syringe.

*Blood samples (0.5—1.0 ml) for ascorbic acid determinations* were taken by heart puncture at the beginning of the experiments, during the operation, and at the end of the experiments. The punctures were made under light ether anesthesia.

2. *Intradermal spread technic.* — Immediately after death the abdominal skin was depilated with the paste described. A rectangular area of the skin was cut out and placed on a wooden block. 0.1 ml of 1 % Evans blue in physiological saline was injected in two (in some cases three or four) symmetrical wheals. In the groups where the effect of hyaluronidase was to be studied, the enzyme was mixed with Evans blue solution immediately before the injection. The concentration of the hyaluronidase (Hyalas, Leo) was 50 V.R.U./ml indicator solution (5 V.R.U. in each wheal). The injections of the indicators were performed slowly. The skin flap was turned with the inside up and fixed to the wooden block with pins. Care was taken not to stretch the skin unduly. The wheals were photographed two, five, ten, fifteen, thirty, and sixty minutes after the injection. In order to prevent the drying of the subcutaneous tissue the whole flap was covered with a layer of paraffin oil. By using the technic described, 6—8 skin-flaps could easily be handled at the same time (Fig. 9).

Measurements were made of the length and width of the ellipsoid areas of the pictures of the wheals at two times magnification. The mean spreading area calculated for each time interval was plotted against the time in minutes.

Some of the wheals were fixed in cold ethanol-methanol mixture, embedded in paraffin and cut in 15 μ to study the distribution of the indicator within the dermal structures.

3. *Perfusion measurements.* — Determinations were made of the rate of flow of a physiological salt solution (Ringer's solution buffered with 0.05 M phosphate buffer at pH 7.0) through isolated connective tissue membranes from scorbutic and normal animals (Groups III b and VII b; see Table 7).

*The apparatus* used for these measurements was constructed by the author, basically in accordance with the equipment outlined by Day

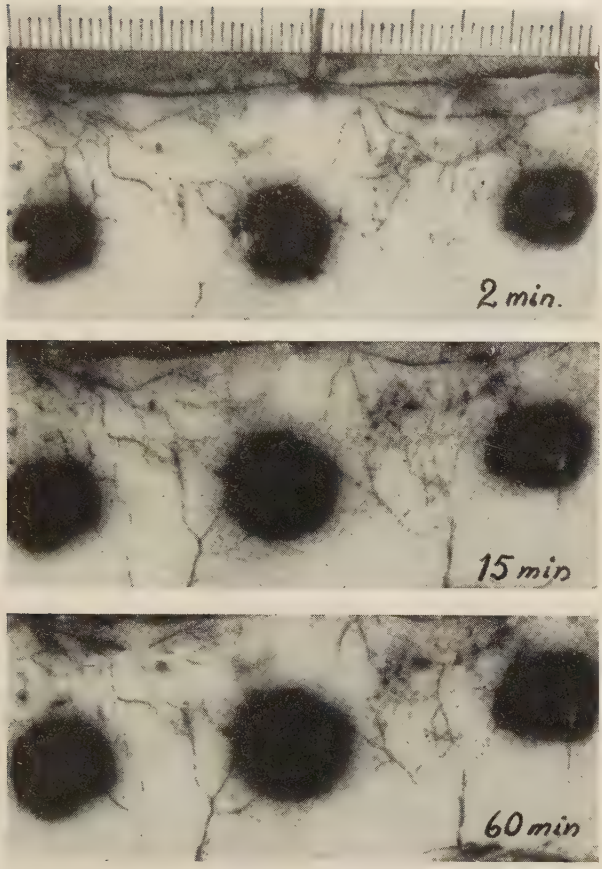


Fig. 9. Intradermal wheals of Evans blue as seen from the inside of the skin (1.3 ×).

(1952) and by Werle and Leusch (1952). The apparatus is shown in Fig. 10.

A 4 ml burette (A) graduated in 0.01 ml is connected to an ordinary three-way stopcock (B) through a Pyrex glass connection. A small hole (C) is bored through the socket and the burette. This canal can be opened and shut by turning the burette so that either the two holes coincide exactly or the inner hole is covered by the socket. When open, the air can enter the burette through the canal. A glass tube (D) with an inner diameter of 5.2 mm is sealed to the lower arm of the stopcock. On this glass tube a rectangular plastic block (E) is mounted so that the glass tube traverses the block and sticks out about half a millimeter. Another plastic block (F) of the same size as that sealed on the stopcock

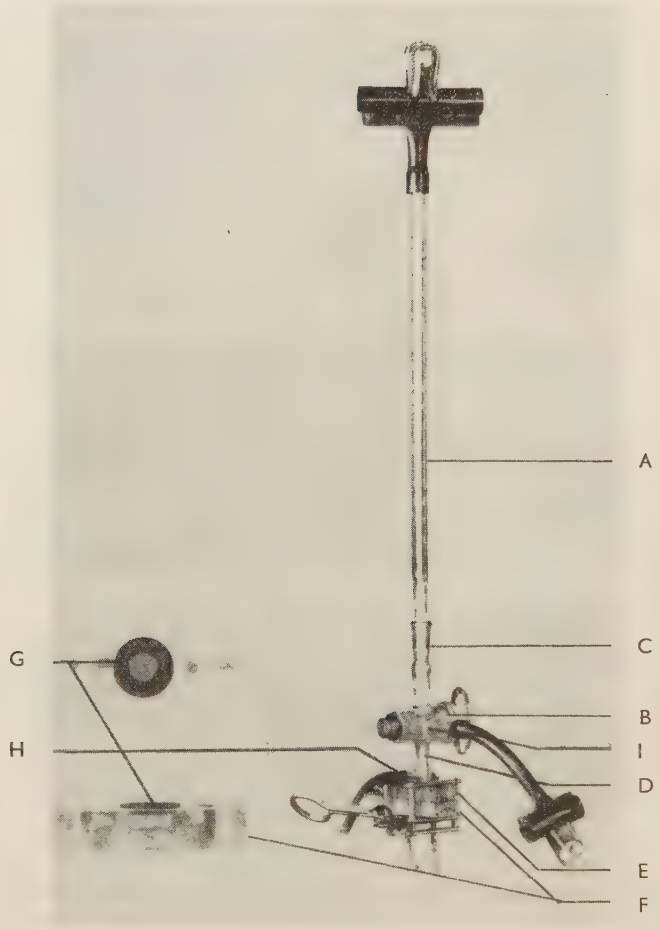


Fig. 10. Apparatus for measurements of flow-rate through isolated connective tissue membranes. For description see the text.

arm and provided with a hole of a diameter of 5.2 mm which fits exactly to the hole of the glass tube, can be attached to the upper block by the aid of a pair of clamps. The lower plastic block carries the membrane which is spread over a small rubber ring (G) whose inner hole has a diameter of 5.2 mm. With the membrane in place the perfusion fluid is introduced into the apparatus through a small glas tube (H) sealed in the lower tube of the stopcock immediately above the plastic block

and, in addition, through the horizontal arm of the stopcock (I). These two tubes, as well as the upper end of the burette, are provided with short pieces of rubber tubings which can be clamped together when the system is to be closed. This system functions as a Boyle-Mariotte's flask with a constant pressure on the membrane which is equivalent to the distance between the air inlet (the small holes in the glass joint) and the membrane. This distance was 80 mm.

*The membranes* used were prepared from the fascia of the thigh. After killing the animal the skin was removed from the hind legs and the fascia was grasped with fine pointed forceps and gently dissected free from the underlying muscles. The rubber ring was pulled in between the muscles and the fascia and the fascia was attached to the ring without being unduly stretched. After being cut along the edge of the rubber ring the fragile tissue could be managed without being damaged. It was stored in a moist chamber until used for measurements. The membrane was adapted for measurements by placing the rubber ring with the attached fascia in a groove made in the lower plastic block and exactly fitting the ring. When pressing the two plastic blocks together the lower end of the glass tubing is pressed against membrane and rubber ring without stretching the tissue.

Fluids containing test substances such as enzymes were brought in direct contact with the membrane without removing the fluid in the burette. This was made possible by closing the communication with the burette; after that the fluid within the chamber between the stopcock and the membrane was removed through the small side tubing (H). The fresh fluid was filled up, the connection with the burette was opened, and the measurements could go on as before.

In order to test the effect of testicular hyaluronidase the enzyme was dissolved in the buffered Ringer solution at 25 V.R.U./ml. 0.2 ml of the enzyme solution was placed in direct contact with the membrane and the measurements of the flow-time were carried on 3 minutes later.

The flow through the membranes was measured at uniform intervals of 3 minutes for at least 15 minutes. 2—3 membranes from each animal were used in these studies. As a whole membranes from 20 animals were measured in the present studies (see Table 11).

After the measurements the membranes (still attached to the rubber rings) were dried in closed chambers containing silicagel for 24 hours at room temperature. The perfunded area of the membranes was stamped out with a circular stamp 5.1 mm in diameter and weighed at a torsion balance at the nearest 0.010 mg. After weighing the membranes were fixed in ethanol-methanol and stained with 0.1 % toluidine blue for histological examination.

4. *Determination of the water content of the skin.* — Small pieces of depilated skin were stamped out with a circular stamp ca. 5 mm in

diameter from an area adjacent to that used for the dermal spread measurements. After weighing the skin pieces were dried in an oven at 100° C for at least 24 hours or until constant weight. Two skin pieces were taken from each animal for these measurements.

5. *Assay of vitamin C.* — Total vitamin C was determined on extracts from whole blood, liver, and adrenals using the dinitrophenylhydrazine procedure of Roe and Oesterling (1944) as modified by Bolin and Book (1947). In this modification of the method dichlorophenolindophenol is used in the place of Norit for the oxidation of ascorbic acid.

In the present investigations the method was further modified with regard to the time of incubation as outlined by Geschwind *et al.* (1951). Instead of incubating the extracts for 3 hr at 37° C the coupling reaction was performed at 58° C for 45 minutes. One hour after the addition of $H_2SO_4$ the color was read in a Beckman DU spectrophotometer at 540 mμ.

Blood as well as liver and adrenals were extracted with 5 % metaphosphoric acid. After centrifuging the extracts they were stored at — 35° C until the determination procedure. The extract of each tissue sample was apportioned to two test tubes, one of which was used as a blank whereas the second portion was oxidized with dichlorophenolindophenol. A standard containing 1 mg% ascorbic acid was always run with the test samples.

Within the range of 1.0—20 γ/ml of ascorbic acid an extinction coefficient of 0.100 corresponds to a concentration of 5.0 γ/ml of the extract used for the determination. This is an average value obtained by running a calibration curve with three independent series of standard solutions of ascorbic acid.

6. *Determination of hexosamines in skin.* — Samples of depilated skin weighing about 1 g were taken from the abdomen of the guinea pigs at autopsy. After cutting the skin flaps in small pieces they were stored in acetone in the cold for about a week. The acetone was changed once or twice during that time. The skin pieces were rapidly taken through distilled water and immediately frozen with carbon dioxide and sectioned on an ordinary freezing microtome at 25 μ. The sections were brought back to fresh acetone and were kept there until the determinations. An appropriate quantity of the skin sections was freed from acetone in vacuo, weighed and hydrolyzed in 3 N HCl for 14 hours at 100° C. A standard containing 0.025 mg glucosamine was run at the same times.

The method of determination used was that of Elson and Morgan as modified by Blix (1948). All determinations were made in duplicate. After preliminary tests the amount of material to be analyzed was adapted to give extinction values of 0.050 to 0.100 which corresponded to 0.015—0.030 mg of hexosamine in the part of the neutralized hydro-

lysate which is acetylated. The values obtained are given as mg of hexosamine in 100 g of aceton-treated, dry tissue.

*7. Histotechnical methods.* — Small pieces from the skin-wound area and from the muscle wounds were taken immediately after killing the animal. The elder-pith pieces were gently dissected free from the underlying muscles and cut out together with the skin covering the implanted cylinders. All the material was fixed in a mixture of ethanol-methanol (1:1) at + 2° C for 16—24 hours. After dehydration in absolute alcohol at room temperature for 4—8 hours they were embedded in paraffin (m. p. 50—52° C) in a special vacuum chamber for 30—60 minutes. The tissues were sectioned at 6—8 μ and stretched on the glass slides with 70 alcohol at 40° C.

From scorbutic animals selected at random the rib junctions, knee-joints, ovaries and part of the adrenals were taken for histological examination. These examinations were performed in order to obtain a biological criterion for the adequacy of the chemical tests.

The following staining methods were used routinely on the whole material:

1) metachromatic staining with an aqueous solution of purified toluidine blue at a concentration of 0.1 % (as described in Part I of this work);

2) periodic acid-Schiff staining (PAS) according to the method devised by Hotchkiss. Treatment with the oxidant was performed for 30 minutes;

3) Hale's method for demonstration of acid mucopolysaccharides with colloidal iron;

4) Hale's method in combination with the PAS staining as devised in Part I (p. 51);

5) Gomori's silver staining for demonstration of reticular fibrils;

6) Heidenhain's "Azan" staining for demonstration of collagen and reticulin, and as a general oversight staining.

In addition, certain sections were stained with 0.2 % *ruthenium red* dissolved in 0.1 M acetic acid and in *Gram's stain* according to the method described by Romeis (1948).

Glycogen was removed from the sections by treatment with malt diastase at 0.5 % dissolved in 0.1 M phosphate buffer at pH 7 for 30—60 minutes at 37° C.

Lipoids were removed from the tissues by treatment with methanol-chloroform (1:1) at 60° C for 1—4 hours.

Certain sections were incubated with testicular hyaluronidase (Hyalas, Leo, 5—10 "viscosity reducing units"—V.R.U.—per ml 0.05 M citrate buffer at pH 6.2 containing 0.15 M NaCl) at 40° C for 1—16 hours before staining with toluidine blue, PAS and Hale's stain. Controls were run in buffer solution only.

## Discussion of the methods

*Adrenalectomy.* — The operative result was carefully controlled at autopsy with respect to the persistence of fragments of the adrenals. In cases where there was reason to assume that the removal of the glands had not been complete, a 5 % solution of acidic silver nitrate was spread over the area surrounding the kidneys. Any remnant of adrenal tissue rapidly reacts with the silver salt forming black precipitates of metallic silver which are easily recognized with the naked eye. No such remnants could be found in any of the adrenalectomized animals in the present material.

*The spreading technic.* — Among the methods used to study the actual condition of the connective tissue ground substance with respect to its state of permeability, the spreading test is the technic most commonly employed. As pointed out by Hayes *et al.* (1950), Linderholm (1951) and others, the validity of the results gained with this method is contingent on adequate control of several technical errors. When the test is performed on living animals such factors as pulsations, movements of the animal, toxicity of the indicator etc. may affect the spread (McMaster and Parsons, 1938; Edlund, 1949; Linderholm, 1951). These factors are however irrelevant in the present studies which are performed on "dead" skin only.

The "initial spread", defined as the increase of the spreading area within the first 10 minutes after the injection of the wheal (Linderholm), is proposed to be essentially determined by the hydrostatic pressure of the injected indicator solution and the resistance offered by the connective tissue. It is of utmost importance, therefore, to perform the injections under strictly standardized conditions. A better reproducibility was obviously achieved when small volumes of indicator solution were injected than when larger volumes were used. In the material recorded in this work 0.10 ml of 1 % Evans blue (dye T-1824) dissolved in 0.8 % saline was injected at a rate of about 0.05 ml/5 sec. The injection pressure produced under these conditions certainly exceeded the "breaking point" of the ground substance (McMaster, 1941; Edlund, 1949) thus causing a derangement of the structural organization of the matrix. The fact that the extent of this derangement varies constitutes an uncontrollable source of error of the method.

The chemical characteristics of the indicator are probably of a definite importance for the spreading mechanism (Edlund, personal communication). The bearing of this statement on the results gained in the present experiments is further discussed below (see page 86).

The error of measurement cannot be estimated when the wheal areas are directly determined with calipers. The photographic method used in the present investigations allows an estimation of this error. On the

basis of duplicate measurements of 40 arbitrarily chosen photos of spread areas the standard error was calculated $\sigma_m = 2.5$[1]. This corresponds to about 2—3 % of the spreading areas found in the present experiments. As seen form the photo in Fig. 9 the boundaries of the wheals were perfectly clear-cut even one hour after the injection of the indicator.

*Perfusion measurements.* — The assay of the flow of physiological saline through an isolated connective tissue membrane may be of value as a complement to the dermal spreading test. The technic used in the present studies is a modification of the one described by Day (1952). It must be emphasized that the possibility of preparing perfectly intact connective tissue membranes is limited indeed. However, the method of preparation of membranes described above seems to be reasonably harmless to the connective tissue structures and may be considered adequate for the present purposes.

The pressure applied upon the membrane—8.0 cm of water—is below the breaking point of *normal* connective tissue, the value of which is about 8.5 cm of water (McMaster, 1941).

The results recorded in the present work are based on measurements made on one single membrane from each animal. This membrane was selected from the two or three available ones on the basis of its microscopical structure and of its permeability as estimated in preliminary tests. Thus, membranes showing obvious defects in their microscopical structure and those containing large vessels, fat deposits etc. were discarded. Nor were membranes used which showed an "abnormal" permeability by perfusion tests. As limiting values for a "normal" permeability were considered flow-rates between 1 µl/min and 100 µl/min. These values were fixed as limiting levels on the basis of average flow values obtained in series of preliminary experiments.

*Assay of vitamin C.* — The procedure of Roe and Oesterling (1944) as modified by Bolin and Book (1947) was followed except that the incubation, as suggested by Geschwind *et al.* (1951), was performed at 58° C for 45 minutes (instead of at 37° C for 3 hours as devised by Roe and Oesterling). The calibration curve run with standard solutions of ascorbic acid ranging from 1.0 to 20 γ/ml was perfectly straight but showed a slope which was less than the one obtained with the original method. The specific extinction *(k)* obtained with the method used in these investigations was 0.200, whereas the value of *k* obtained with the original method was 0.250.

The error of measurement was calculated from duplicate determinations made on 40 samples of blood taken from animals at the beginning of the experimental time. The standard error was calculated to

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[1] According to the formula $\sigma_m = \sqrt{\frac{\sum d^2}{2n}}$ (after Dahlberg, 1940).

$\sigma_m = 0.03$. Thus, practically no estimation deviates more than 0.1 mg % from the real value.

*Determination of amino sugar in skin homogenates.* — The method of Elson and Morgan as modified by Blix (1948) was used. The skin samples were "homogenized" by cutting 25 μ sections with an ordinary freezing microtome. 3 N HCl was used for hydrolysis of the material since preliminary tests had shown that the most reproducible results were gained with this concentration of the acid. Blanks were obtained by using the acid alone.

The error of measurement was calculated on the basis of 25 arbitrarily chosen duplicate determinations. It was found to be 7.9. Thus, practically no single determination deviates more than 24 mg % from the real value. The values recorded in this work are the mean of duplicate determinations; the error in this case will be $7.9/\sqrt{2}$ or 5.6, which means that the error of measurement only amounts to about 1—2 % of the hexosamine values recorded in the present material.

*Histotechnical methods.* — It is a well-known fact that most histochemical methods are highly dependent upon the various treatments preceding the staining procedure. The first requirement is that the substances to be indicated must be immobilized at their native sites. Secondly, the fixation must not damage or change the substances in a way that masks or abolishes their reactivity towards the staining reagents to be used. The freezing-drying method offers great advantages in these respects, but it should not be considered the only saving method for all purposes.

In a series of preliminary experiments on a material similar to that recorded here, the histochemical results gained with frozen-dried specimens were compared with those obtained after fixation with ethanol-methanol as performed in the present work. The freezing-drying was performed either *in vacuo* (according to the Altmann-Gersh method) at —41° C and $10^{-4}$ mm Hg, or in chilled ethanol-methanol according to the method described by the author (Persson, 1952). I could not find that the freezing-drying methods offered any obvious advantages when compared to the ordinary fixation in alcoholic medium at + 2° C as far as the preservation and staining of the connective tissue constituents were concerned. It must be remembered that possible advantages of a certain method of fixation may be more or less completely abolished by the treatment interposed between the fixation and the staining procedure.

The use of *ruthenium red* (ammoniated ruthenium oxychloride) as a stain for demonstration of acid mucopolysaccharides within connective tissues has not been reported in the modern histological literature.

Mangin (1893) reported the use of ruthenium red for visualizing pectin within vegetables. Nicolle and Cantacuzène (1893) noticed that the intercellular substance of cartilage, sclera and cornea as well as mast cell granules took a brilliant red stain with acidified ruthenium red. The use of the stain in this work was initiated by the finding that it was a rather good indicator of the presence of hyaluronic acid and chondroitin sulfuric acid within filter paper strips used for separation of these substances with the paper electrophoresis method (unpublished work).

The staining schedule routinely used was as follows: staining of the deparaffinized sections in 0.2 % ruthenium red dissolved in 0.1 M HAc for 15 minutes, followed by differentiation in 0.1 M HAc in 70 % ethanol for 15 minutes, and dehydration and mounting in balsam. With this method hyaluronic acid as well as chondroitin sulfuric acid and heparin take a rather strong color varying from bright red to brown reddish. When tested on purified substances, the color of the hyaluronic acid sample employed (from umbilical cord) was brown red whereas chondroitin sulfuric acid and heparin took bright red to red violet colors. Such distinct differences were not found within tissue sections.

*Gram's stain* (crystal violet differentiated with Lugol's iodine solution) is considered a general reagent for *conjugated proteins* (Lennox *et al.*, 1952). A positive reaction requires a sufficiently basophilic non-protein fraction united to a protein fraction. This stain would thus be able to indicate the presence of "intact" mucopolysaccharide-protein complexes within connective tissue.

Inasmuch as no bacterial hyaluronidase was available to me, purified *testicular hyaluronidase* (Hyalas, Leo) has been used for digestion of hyaluronic acid in the sections. This enzyme hydrolyzes chondroitin sulfuric acid as well, but at a slower rate than hyaluronic acid (Meyer *et al.*, 1941). According to Meyer and Rapport (1951) the chondroitin sulfuric acid present in skin (ChS-B) is resistant to testicular hyaluronidase. Heparin is not digested with testicular hyaluronidase. When tested on sections from umbilical cord the enzyme employed more or less completely destroyed the metachromatic potency of the Wharton jelly within 2—4 hours at 40° C, whereas the metachromasia within the big vessels is practically intact after this treatment. *Though no absolute specificity can be attributed to histochemical action of testicular hyaluronidase it seems justified to presume that a comparatively short digestion with the enzyme gives more reliable results with respect to the specific action on hyaluronates.*

CHAPTER VI

# Results

## Progress of vitamin C deficiency

The animals on the scorbutogenic diet (Groups III a—VI and Group IX) gained weight during the first 7—10 days of the experiment. During that time they appeared healthy and active. The growth curve then reached a plateau and there was a beginning failure of appetite. In the third week body weight began to decline and then fell rapidly until death. At the end of the experiments the animals were listless and apathetic, showed a gradual swelling and tenderness of the joints, gingival hemorrhages and diarrhoea.

The development of scurvy in the animals treated with *cortisone* (Group IV) seemed to be somewhat modified. These animals showed a decrease in body weight but the symptoms from the joints were less significant and they did not grow lethargic during the last part of the experimental period like the other scorbutic animals.

*Adrenalectomy,* by contrast, significantly aggravates the scurvy, and a number of animals died during one week after the operation. This was not the case when adrenalectomy was performed on adequately fed guinea pigs (Group VIII c), *nota bene* when discounting the animals which succumbed to the surgical intervention.

A significant improvement in the general symptoms of scurvy could be observed in accession to *sham-operation* (Group III a) and after making the circular skin wounds in the animals of Group IX. This improvement was however of short duration.

The addition of 0.1 mg of ascorbic acid per day to the animals in Groups I and II obviously had some influence on the development of the scurvy inasmuch as these animals never showed such pronounced symptoms of the disease as the animals fed the basal diet only.

The guinea pigs receiving 10 mg of ascorbic acid per day in addition to the basal diet (Groups VII a—VIII c) gained weight normally and appeared healthy and active. Adrenalectomy performed on these animals (Groups VIII b and VIII c) was followed by a moderate decrease in body weight.

The data for the average body weights and the concentrations of

Table 8. *Body Weights and Tissue Ascorbic Acid Concentrations of the Animals at Start of Experiments, at Operation, and at Autopsy.* ($M \pm \varepsilon_M$).

| Group | No. animals | At start | | At operation | | | At autopsy | | | | Gross signs of scurvy |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Body weight g | Blood asc. acid mg % | Body weight g | Blood asc. acid mg % | Adren. asc. acid mg % | Body weight g | Blood asc. acid mg % | Adren. asc. acid mg % | Liver asc. acid mg % | |
| I | 10 | 372 ± 11 | 1.07 ± 0.10 | 361 ± 12 | 0.63 ± 0.05 | — | 316 ± 10 | 0.25 ± 0.03 | — | — | + |
| II | 10 | 288 ± 13 | 0.93 ± 0.07 | 269 ± 5 | 0.38 ± 0.04 | — | 250 ± 9 | 0.21 ± 0.02 | — | — | + + |
| III a | 24 | 363 ± 10 | 0.91 ± 0.03 | 344 ± 9 | 0.43 ± 0.02 | — | 285 ± 9 | 0.19 ± 0.01 | — | 1.22 ± 0.08 | + + + |
| III b | 20 | 341 ± 12 | 0.77 ± 0.04 | — | — | — | 233 ± 11 | 0.12 ± 0.01 | 1.58[1] ± 0.24 | 0.86 ± 0.02 | + + + |
| IV | 25 | 363 ± 11 | 0.79 ± 0.03 | 338 ± 12 | 0.37 ± 0.02 | | 285 ± 8 | 0.16 ± 0.01 | 3.67[2] ± 0.40 | 1.79 ± 0.19 | + + |
| V | 16 | 378 ± 23 | 0.75 ± 0.03 | 315 ± 11 | 0.31 ± 0.03 | 8.1[3] ± 1.4 | 262 ± 22 | 0.10 ± 0.02 | — | 0.80 ± 0.07 | + + + |
| VI | 6 | 401 ± 9 | 0.97 ± 0.05 | 356 ± 14 | 0.32 ± 0.03 | 8.4 ± 2.0 | 316 ± 15 | 0.19 ± 0.02 | — | 1.86 ± 0.10 | + + + |
| VII a | 8 | 382 ± 19 | — | 399 ± 24 | — | — | 410 ± 29 | 1.02 ± 0.13 | — | 23.5 ± 4.3 | o |
| VII b | 20 | 325 ± 16 | — | — | — | — | 381 ± 11 | 0.83 ± 0.03 | 71.7 ± 5.7 | 17.4 ± 1.4 | o |
| VIII a | 6 | 292 ± 9 | 0.75 ± 0.02 | 342 ± 29 | 0.85 ± 0.03 | — | 317 ± 24 | 0.80 ± 0.06 | 67.6 ± 4.3 | 16.7 ± 2.3 | o |
| VIII b | 6 | 343 ± 7 | 0.64 ± 0.01 | 354 ± 7 | 0.64 ± 0.07 | 55.3 ± 6.0 | 286 ± 4 | 0.70 ± 0.08 | — | 34.8 ± 4.3 | o |
| VIII c | 6 | 413 ± 16 | 0.80 ± 0.03 | 431 ± 20 | 0.75 ± 0.04 | 77.2 ± 9.2 | 388 ± 15 | 0.39 ± 0.03 | — | 11.6 ± 1.4 | o |
| IX | 16 | 242 ± 5 | 0.79 ± 0.04 | 207 ± 4 | 0.17 ± 0.02 | — | 188 ± 5 | 0.08 ± 0.02 | — | 1.36 ± 0.20 | + + + |

[1] Adrenals from 12 animals.
[2] Adrenals from 17 animals.
[3] Adrenals from 11 animals.

ascorbic acid (including dehydroascorbic acid and diketogulonic acid) found in whole blood, liver and adrenals are listed in Table 8.

Although the average values of ascorbic acid concentration in whole blood are low in the scorbutic groups (Groups III a—VI), the single values very seldom reached zero even in the premortal phase of the

disease. In a few tests performed on serum from such severely affected guinea pigs no ascorbic acid could be found.

The liver and the adrenals retained their ascorbic acid to a certain extent even in animals that succumbed to scurvy.

At autopsy all the animals on the scorbutic diet showed swollen wrists and knee-joints with large hemorrhages within the muscles surrounding the joints. The bones were fragile and the incisor teeth had loosened from the alveolar bone. Bleedings were also found in the intestinal canal and here and there in the muscles of the trunk. The animals given small dosages of ascorbic acid (Groups I and II), like those treated with cortisone (Group IV), showed on an average a smaller extent and degree of the scurvy lesions.

None of the animals given adequate dosages of ascorbic acid in addition to the basal diet showed any signs of macroscopical or microscopical scurvy.

*Discussion.* — According to Kuether *et al.* (1944) the determination of the ascorbic acid content of the *whole blood* is the best method for evaluating the nutritional status of an animal with respect to vitamin C. These authors state that at a whole blood concentration of 0.25 mg % the various tissues achieve a considerable storage of vitamin C. To maintain such a whole blood concentration an intake of about 1.23 mg of ascorbic acid per 100 g of body weight per day is necessary. These data are in agreement with those found in the present studies. The authors mentioned moreover showed that at an ascorbic acid blood level above 0.22 mg/100 ml no signs of scurvy are observable even microscopically.

## Permeability of dermal connective tissue and muscle fascia

*Dermal spread of Evans blue.* — As seen from Tables 9 a—b and in Figs. 11—12 the spread of Evans blue injected into the dermal connective tissue was significantly increased in scorbutic animals (Fig. 12, Curve $E_s$) when compared to normal animals (Fig. 11, Curve $E$). The areas of spread indicated in the tables and figures are computed on wheals injected in abdominal skin flaps. In some series wheals were injected in the dorsal skin at the same time as the ordinary wheals were made in the abdominal skin. The same range of difference between the scorbutic and normal animals was found in these tests.

*The effect of testicular hyaluronidase* on the rate and extent of spreading is shown in Tables 9 a—b and in Figs. 11 and 12. In the skin of normal animals the enzyme had a pronounced enhancing effect on the spread of the indicator (Fig. 11, Curve $E + H$) whereas such an effect was practically absent in the scorbutic animals (Fig. 12, Curve $E_s + H$).

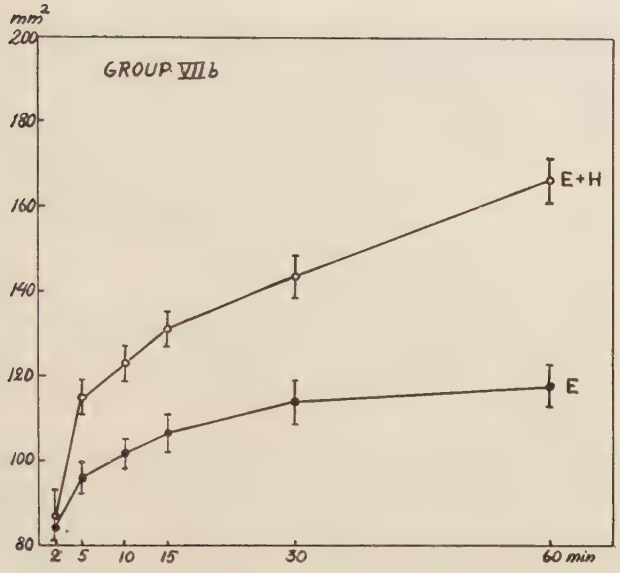


Fig. 11. Mean spreading area in normal guinea pigs.
E = Evans blue; H = Hyaluronidase; (Standard errors of the means are indicated).

The spreading of Evans blue found in the scorbutic animals was nearly identical with the spread of the indicator plus hyaluronidase injected into normal skins (compare Curve $E_s$ in Fig. 12 and Curve $E + H$ in Fig. 11).

As seen in Fig. 12 (Curve $E_c$) and Table 10 *cortisone* exerted some depressing effect on the spread of Evans blue in scorbutic animals. However, this inhibiting effect is significant only in the 60-minute spreading areas. The cortisone-treated animals had received 5 mg of the hormone per 100 g of body weight once a day for 6—8 days before the spreading test was performed. Furthermore, it must be taken into account that these animals were all "sham-operated" on the day of the first injection of cortisone. Probably they were not in all respects comparable to the non-operated animals in Group III b (see Table 7 and Fig. 12).

In order to obtain information on the localization of the indicator within the dermal tissues, a number of wheals from animals in the various groups concerned were sectioned and examined under the microscope. In all the cases investigated the indicator was spread out within the corium between the epidermis and the cutaneous muscle. As a rule, however, penetration of the indicator had taken place through the cutaneous muscle down in the subcutaneous tissue. Apparently this

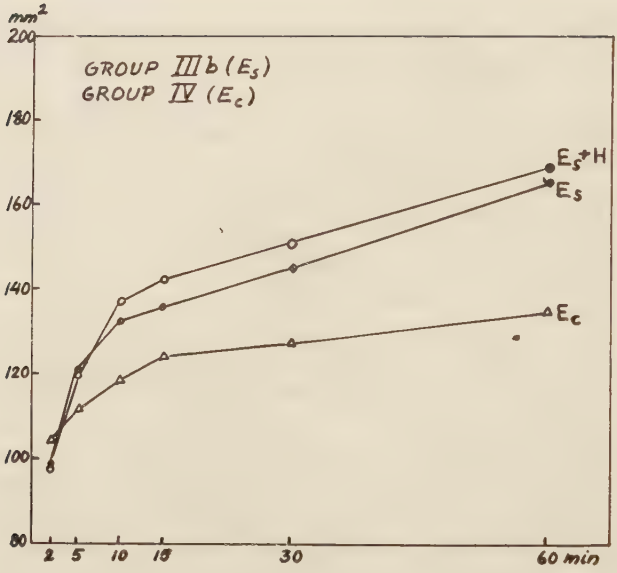


Fig. 12. Mean spreading area in scorbutic guinea pigs ($E_s$, $E_s+H$) and in scorbutic guinea pigs treated with cortisone ($E_c$).
E = Evans blue; H = Hyaluronidase.

penetration followed the vessels and their surrounding connective tissue sheaths through the muscle. The amount of indicator disappearing this way seemed to be fairly small.

The derangement of the structure of the corium layer caused by the high initial pressure applied on the tissue during the injection of the indicator was of a varying extent in the specimens studied microscopically.

Within normal skin the form of the indicator wheal as seen on a section cut perpendicularly to the epidermal layer was essentially that of a cone with the base lying on the cutaneuos muscle and the cut off-apex facing the epidermis. The wheals containing hyaluronidase, on the other hand, apparently spread out in a more diffuse way filling out the whole area between the epidermis and the cutaneous muscle. Similar pictures were observed when Evans blue (without hyaluronidase) spread within the corium of scorbutic animals. The texture of the scorbutic skin samples was on an average looser than that found in normal skin.

*Perfusion of Ringer's solution through isolated muscle fascia.* — As seen from Table 11 the flow-rate of the Ringer solution through the membranes from scorbutic animals was higher (on average 40 µl/min)

Table 9 a. *Dermal Spread of Evans Blue in Normal and Scorbutic Guinea Pigs. Effect of Testicular Hyaluronidase on the Spread.*

| Time after injection of the wheal | Normal animals (n=20) Group VII b | | Scorbutic animals (n=20) Group III b | |
|---|---|---|---|---|
| | Evans blue E | Evans blue + Hyalas E+H | Evans blue $E_s$ | Evans blue + Hyalas $E_s$+H |
| 2 mins. | 84.1±2.8 | 87.0±3.2 | 98.2±3.2 | 97.4±3.8 |
| 5 mins. | 96.2±3.9 | 115.6±6.2 | 120.8±4.3 | 117.9±5.6 |
| 10 mins. | 101.6±4.5 | 123.7±6.0 | 133.3±4.6 | 127.1±6.4 |
| 15 mins. | 106.8±6.3 | 131.4±5.7 | 135.3±4.3 | 134.0±7.5 |
| 30 mins. | 114.2±5.6 | 144.4±7.2 | 145.6±5.5 | 142.8±7.8 |
| 60 mins. | 117.8±7.6 | 167.7±7.3 | 165.7±5.5 | 168.1±8.4 |

Table 9 b. *Differences between the Groups Indicated in Table 9 a.*

| Time after injection of the wheal | Diff. E–(E+H) | Diff. E–$E_s$ | Diff. (E+H)–$E_s$ | Diff. $E_s$–($E_s$+H) |
|---|---|---|---|---|
| 2 mins. | − 2.9± 4.2 | −14.1±4.3 | −11.2±4.5 | 0.8± 5.0 |
| 5 mins. | −19.4± 7.3 | −24.6±5.8 | − 5.2±7.5 | 2.9± 7.1 |
| 10 mins. | −22.1± 7.5 | −31.7±6.4 | − 9.6±7.6 | 6.2± 7.9 |
| 15 mins. | −24.6± 8.5 | −28.5±7.6 | − 3.9±7.1 | 1.3± 8.6 |
| 30 mins. | −30.2± 9.1 | −31.4±7.8 | − 1.2±9.1 | 2.8± 9.5 |
| 60 mins. | −49.9±10.5 | −47.9±9.4 | 2.0±9.1 | −2.4±10.0 |

Table 10. *Effect of Cortisone on Dermal Spread in Scorbutic Guinea Pigs.*

| Time after injection of the wheal | Scorbutic animals (n=20) $M \pm \varepsilon_M$ | Scorbut + Cortisone (n=20) $M \pm \varepsilon_M$ | Diff. |
|---|---|---|---|
| 2 mins. | 98.2±3.2 | 103.6±4.1 | − 5.4±5.2 |
| 5 mins. | 120.8±4.3 | 111.9±4.8 | 8.9±6.4 |
| 10 mins. | 133.3±4.6 | 118.6±5.0 | 14.7±6.8 |
| 15 mins. | 135.3±4.3 | 124.3±4.8 | 11.0±6.4 |
| 30 mins. | 145.6±5.5 | 127.6±4.6 | 18.0±7.2 |
| 60 mins. | 165.7±5.5 | 135.0±3.2 | 30.7±6.4 |

than was the flow-rate through the normal membranes (about 16 µl/min).

The effect of hyaluronidase on the permeability of the normal membranes was remarkable. The velocity of flow through these membranes increased after treatment with the enzyme to about three to four times its initial rate. No such pronounced effect of hyaluronidase was found

Table 11. *Flow-Rate of Buffered Ringer's Solution through Muscle Fascia from Scorbutic and Normal Animals. Action of Testicular Hyaluronidase (Hyalas). (Group III b = scorbutic animals; Group VII b = normal animals.)*

| Animal | Group | Flow-rate without Hyalas µl/min. | Flow-rate with Hyalas µl/min. | Increase of flow-rate % | Membrane weight µg |
|---|---|---|---|---|---|
| 1 | III b | 35 | 44 | 26 | 50 |
| 2 | » | 32 | 32 | 0 | 40 |
| 3 | » | 29 | 37 | 28 | 40 |
| 4 | » | 19 | 25 | 32 | 90 |
| 5 | » | 44 | 65 | 48 | 40 |
| 6 | » | 44 | 51 | 16 | 50 |
| 7 | » | 35 | 36 | 3 | 60 |
| 8 | » | 29 | 37 | 28 | 60 |
| 9 | » | 53 | 59 | 11 | 40 |
| 10 | » | 82 | 93 | 13 | 30 |
| 1 | VII b | 28 | 127 | 354 | 40 |
| 2 | » | 12 | 46 | 283 | 50 |
| 3 | » | 5 | 61 | 1 120 | 50 |
| 4 | » | 11 | 20 | 82 | 40 |
| 5 | » | 7 | 14 | 100 | 60 |
| 6 | » | 3 | 17 | 467 | 40 |
| 7 | » | 51 | 215 | 322 | 40 |
| 8 | » | 8 | 33 | 313 | 60 |
| 9 | » | 4 | 11 | 175 | 50 |
| 10 | » | 32 | 76 | 138 | 40 |

on "scorbutic membranes." In these cases the flow-rate increased only about 20 % after the treatment with the enzyme.

The velocity of flow through the different membranes within the same group showed significant variations especially concerning the normal membranes (Group VII b). This is explained by the lack of uniformity of thickness and structure of the various membranes. As seen in the table the weights of the dried membranes were fairly uniform. Such a uniformity was, however, not apparent when weighing was performed on membranes which had not been treated with hyaluronidase (not recorded in the table).

Under the conditions described the velocity of flow through the membranes very rapidly reached a steady state showing insignificant variations between the 3-minute measurements.

*Water content of the skin.* — With the method used in the present experiments a constant weight of the skin samples was mostly obtained after drying for 24 hours.

The results of the determinations are summarized in Table 12. Obviously the skin of the scorbutic guinea pigs contained significantly more moisture than could be found in the normal skins.

Table 12. *Water Content of Skin in Normal and Scorbutic Guinea Pigs. Effect of Cortisone on the Water Content of the Skin in Scorbutic Animals.*

| Animals | No. animals | Moisture content in % of fresh weight $M \pm \varepsilon_M$ | Diff. |
|---|---|---|---|
| Normal animals ..... Group VII b | 20 | 68.4 ± 1.0 | 5.8 ± 1.9 |
| Scorbutic animals..... Group III b | 20 | 74.2 ± 1.6 | 5.8 ± 2.2 |
| Scorbutic animals + Cortisone Group IV | 20 | 68.4 ± 1.5 | |

Table 13. *Hexosamine Content of Skin Samples from Normal and Scorbutic Guinea Pigs and from Scorbutic Guinea Pigs Treated with Cortisone.*

| Animals | No. animals | Hexosamin mg/100 g dried skin | Diff. |
|---|---|---|---|
| Normal animals ..... Group VII b | 18 | 447.8 ± 9.8 | 74.9 ± 13.0 |
| Scorbutic animals..... Group III b | 20 | 522.7 ± 8.6 | 118.5 ± 17.3 |
| Scorbutic animals + Cortisone | 15 | 404.2 ± 15.0 | |

According to the data recorded in the table cortisone in some way depressed the water-binding capacity of the scorbutic skin. However, this effect is not significant in the present material.

*Hexosamine content of the skin.* — The hydrolysis as performed in the present investigations caused a complete resolution of the "homogenized" skin samples. The yellow-brown color of the hydrolysate was easily removed by shaking with a small portion of carbon and filtering. The acetylation was performed at pH 9.3—9.6. The concentration of hexosamine within the skin was calculated in mg per 100 g dried, aceton-treated tissue. The results are summarized in Table 13.

According to the data given in the table the hexosamine content of the skin from scorbutic animals is significantly increased when compared to the values obtained in normal skins. Apparently cortisone exerted a "normalizing" effect on the scorbutic dermal tissue with respect to its content of amino sugar. No significant difference was found in the hexosamine content of normal skins as compared to skin samples from scorbutic animals treated with 5 mg of cortisone per 100 g of body weight once a day for 6 to 8 days.

## Discussion

The results obtained in the present investigations show that *the barrier-effect of the connective tissue ground substance is significantly weakened in scurvy.* It is generally assumed that the barrier-effect is mainly dependent on the quantity and degree of aggregation of hyaluronic acid within the tissue (Duran-Reynals, 1942; Meyer, 1945). The increased permeability of the loose connective tissues in scurvy might be due to either quantitative or qualitative changes of the ground substance complex and especially its mucopolysaccharide constituents. A diminution of the interfibrillar gel would be consistent with the assumption that there is a decreased formation or a failure of maintenance of the matrix in connective tissues of scorbutic animals (Wolbach and Howe, 1926; Dalldorf and Zall, 1930; Boyle *et al.*, 1940). Such an interpretation, however, does not seem to be consistent with the finding of an increased amount of amino sugar as recorded in the present work.

Hexosamine is an integrant part of the acid mucopolysaccharides found in connective tissue and may as such be used as an indicator of the approximate amount of these substances within the skin. Thus it may be concluded that *the amount of mucopolysaccharides was not diminished in the skin of the scorbutic animals but rather increased.* The weakening of the barrier-effect of the dermal connective tissue may thus be implicated in a *qualitative change of the ground substance complex,* probably depending on a depolymerization and/or disaggregation of the complex.

The diminutive effect exerted by testicular hyaluronidase on the dermal spread of Evans blue as well as on the flow-rate of Ringer's solution through isolated muscle fascia gives further support to the assumption that the acid mucopolysaccharides of the ground substance are qualitatively changed—depolymerized and/or disaggregated—in scurvy. Consequently, the "barrier-breaking" action of hyaluronidase may be insignificant when the barrier (primarily the hyaluronic acid) is more or less broken down already.

As regards the influence of the chemical composition of the indicator substance on the spreading mechanism, this factor seems to be of no consequence in the present experiments. A series of control tests was performed with the use of India ink injected into skin flaps adjacent to those used for the spreading tests recorded in this work. The results gained with this indicator were in all respects consistent with the findings reported in the present paper. Nevertheless, a somewhat faster rate of spread was noticed with the India ink in both the normal and scorbutic animals. This could be explained by the fact that Evans blue is an acid dye possessing a certain degree of affinity to the acidophilic

components of the connective tissue. Thus the spreading of this dye might be somewhat influenced by the adsorption of part of the dye to positively charged groups accessible within the structures. The excess of the dye is so large, however, that the effect of such an adsorption may be neglected. In this connection it may be mentioned that Gersh and Catchpole (1949) found that Evans blue intravenously administered to living animals is selectively taken up by depolymerized, water-soluble ground substance. The mechanism of this staining is obscure. It may be suggested that the reaction depends on a release of acidophilic protein components when the carbohydrate-protein complex is disaggregated. In any case, in the present material no significant differences between normal and scorbutic animals could be found in the degree and extent of adherence of Evans blue to the interfibrillar substance within the wheals examined microscopically. By washing the "wheal sections" in 70 % alcohol or in distilled water most of the indicator was dissolved from the sections. The amount of stain retained within the structures was apparently of the same magnitude in the various groups concerned.

The increased *velocity of flow through the scorbutic membranes* as compared to the normal ones may possibly be due to a lowering of the breaking point of scorbutic connective tissue. In such a case the pressure applied on the membranes (8 cm of water) may exceed that point and thus cause derangements of the interfibrillar, submicroscopical structures. If the theory of Day (1952) is taken for granted, such a derangement would imply the break-down of the protein network of the ground substance. The fascia would in this case no longer act as a semipermeable membrane but rather as a strainer made up of collagenous fibers and offering very small resistance to the Ringer solution. In order to examine this possibility a few experiments were performed on scorbutic membranes showing a high initial velocity of flow (between 75 and 100 µl/min). After perfusion at the ordinary pressure of 8 cm of water, the lower end of the apparatus (below the three-way stopcock; see Fig. 10) was dipped into Ringer's solution, the level of which was rised to about 3 cm above the membrane. In this way the pressure on the membrane was reduced to about 5 cm. The flow-rate at this pressure was about the same as that found initially. According to McMaster (1941) and Edlund (1949) this finding may be interpreted as proving that the structure of the ground substance is intact. Otherwise the flow-rate would have decreased significantly when the pressure was lowered.

The difference in the condition of the ground substance within the fasciae of normal and scorbutic animals could be demonstrated in another way. When perforating a normal fascia with a very fine glass-needle (about 50 µ thick) during the perfusion test, a considerable increase of flow-rate was immediately noticed. This quickening of the

velocity of flow was however of a short duration. In a few minutes the flow-rate returned to the initial value. Such a stopping of a mechanically produced leak could not be observed in the scorbutic membranes, probably due to lack of sufficiently highly polymerized or aggregated material in the interfibrillar ground substance.

*Histological examination* of the membranes after the perfusion procedure (including treatment with hyaluronidase) revealed no significant structural differences between the membranes of the two experimental groups concerned. In some of the normal membranes traces of metachromatically stained ground substance could be observed at the periphery of the circular fascia samples. Samples of normal membranes which had not been used for perfusion measurements showed a pronounced metachromatic staining of the interfibrillar areas.

The increased *water-binding capacity* of the dermal connective tissue of the scorbutic guinea pigs may be correlated to an increased amount of the extremely hydrophilic mucopolysaccharides. Similar correlations between water content and hexosamine concentration have been reported by Ludwig *et al.* (1950) in studies on experimental exophthalmos.

## Histological series

For the study of the granulation tissue around the implanted elderpiths and of the processes of repair in the skin and muscle wounds, 2—6 animals from each experimental group recorded in Table 7—with the exception for Groups III b and VII b—were killed on the 2nd, 5th and 8th day after operation. In Groups I and II some animals were killed, in addition, on the 10th, 12th and 14th day after operation.

*Normal animals: Group VII a.* — Already on the 2nd day after operation there was a lively migration of fibroblasts from adjacent tissues into the wound areas. The cells were surrounded by ground substance which was partly amorphous and partly showed a fibrous structure (Plate II: 3). This ground substance took a strong metachromatic color with toluidine blue and was in addition stained with Hale's colloidal iron and ruthenium red. The PAS reaction was practically negative but with the silver impregnation method of Gomori minute silver granules could be seen in the intercellular spaces. This method also revealed the presence of delicate fibrils running along the borders of the cells. The fibroblasts showed a great variety of forms, some being elongated and slender while others were triangular or rounded (Plate II: 3).

Around the implanted elder-piths fibroblasts showed a great activity. A certain difference was observable between the cells surrounding the hyaluronidase-containing plate and those covering the "control plate". Obviously the enzyme affected the cellular elements which showed a marked hyperplasia and in addition exhibited a vacuolated and swollen appearance, their cytoplasm containing inclusions which were non-metachromatic but slightly PAS-positive. The ground substance within the area nearest to the elder-pith was non-metachromatic and Hale-positive, whereas at some distance from the implanted plate patches of strongly metachromatic matrix could be seen.

By the 5th day the fibroblasts were embedded in a network of delicate reticulum fibrils (Plate II: 1—2) the meshes of which were more or less filled up with ground substance showing a strong metachromasia (Plate 5: 7) but a moderate Hale-reaction (Plate 5: 6). At this time no significant effect of the hyaluronidase could be found in the tissue surrounding the implanted enzyme-containing elder-pith.

In some places within the wound area elongated fibroblasts could be observed embedded in a matrix of mainly amorphous appearance which was penetrated by gracile fibrous structures some of which apparently adhered to the cell-body (Plate II: 4). These fibrils, seemingly spun off the cytoplasm, took a strong metachromatic stain and were furthermore PAS-positive and could be impregnated with silver. Moreover, they were partly Gram-positive but were hardly visible with aniline blue (Azan). These staining reactions indicate that these fibrils must be interpreted as newly formed reticulum fibrils containing, or surrounded by, acid mucopolysaccharide.

On the 8th day after operation the fibroblasts were more uniformly elongated and orientated (Plate II: 5). The ground substance showed a fibrous apperance and coarser bundles of reticulin as well as the formation of collagenous fibers. These structures showed a comparatively strong PAS reaction (Plate II: 6). The wound area of the skin incision was filled with orientated, fibrous connective tissue (Plate I: 1) and the elder-piths were surrounded by a dense, collagenous capsule.

*Scorbutic animals: Group III a.* — On the 2nd day after operation there was a prominent fibroblastic proliferation to be seen in the wound areas. The cells resembled mesenchymal cells and were pleomorphic. Hemorrhages of varying extent were observed within the wound areas and around the implanted elder-piths. No effect of the hyaluronidase could be found in the tissue surrounding the enzyme-containing plate.

The abnormal appearance of the fibroblasts was quite prominent in sections taken on the 5th day after operation (Plate III: 4—7). Their size and form varied, some of the cells being small with a scanty amount of cytoplasm and others showing a large nucleus surrounded

by a vacuolated cytoplasm. When stained with toluidine blue no traces of extracellular metachromasia could be found within the sections (Plate III: 6). In fact, the spaces between the cellular elements were apparently empty. This is not so, when the PAS staining (Plate III: 4) or Hale's method (Plate III: 5) is used. In these sections the "empty" extracellular areas are then partly filled with a pink, often granular substance. Furthermore, the cytoplasm of the cells is more or less packed with heavily stained granules. In most specimens examined there were but few reticular fibrils to be seen at this stage of repair. Moreover, these fibrils showed a chaotic arrangement and were often irregular in thickness.

By the 8th day no further attempts at repair seemed to be made. When staining the sections with the combined Hale-PAS method a comparatively large amount of Hale-positive extracellular material could be distinguished (Plate III: 7). As seen in the figure the cells were crowded with granules which took a strong reddish color with the Schiff reagent whereas the extracellular material retained its Prussian blue. Swollen, irregular masses of collagen penetrated the scanty amounts of granulation tissue surrounding the elder-piths (Plate III: 1—3). The skin wound was covered with a thin layer of epidermal cells, but under this layer the healing was imperfect (Plate I: 3) showing very few orientated fibrils but often large non-organized hemorrhages.

The ground substance made visible with the aid of the Hale reaction or partly by staining with the PAS reagents is readily abolished by treating the sections with testicular hyaluronidase for 4 hours. This, however, would not seem to be due to an enzymatic effect, for the same or approximately the same effect is observed within the control sections incubated in buffer solution only. Thus the abolishment of the intercellular substance may be explained by its solubility in the buffer solution. When sections from normal animals were treated with buffer solution only, no effect could be observed on the stainability of the ground substance whereas the metachromatic reaction of this substance was practically absent after treatment with hyaluronidase for 4 hours.

It may be pointed out that the ground substance in the scorbutic animals always showed an amorphous or granular appearance. The characteristic fibrous precipitates found in sections from normal animals were not observed in scurvy.

*Partially ascorbic-acid-depleted animals: Group I.* — The histological pictures obtained from the animals in this group resemble those described above (Group III a). In some of the animals, however, a scanty amount of metachromatic ground substance could be observed at the periphery of the wound area. Moreover, a tendency of fiber

formation could also be noticed in the metachromatic areas. No mature collagen could however be found. The fibroblasts often showed a pronounced metachromasia of their cytoplasm which was unaffected by treatment with testicular hyaluronidase. This metachromasia might be due to nucleic acids. Since no ribonuclease was available to me, this assumption could not be verified. As a whole the fibroblasts in this group did not exhibit such pronounced abnormalities as those found in the foregoing group.

*Effect of cortisone on the reparative processes in scurvy: Group IV.* — It must be stressed that the animals in this group were treated with cortisone from the day of operation until being killed. Accordingly, the total amount of the hormone given to the animals used for histological examinations varies according to the length of the experimental time in each case.

Already on the 2nd day after operation a pronounced effect of the hormone could be demonstrated in the sections. The non-retarded proliferation of fibroblasts seen in the ordinary scorbutic animals seemed to be regulated by the action of cortisone. The cells were more uniform in size and appearance, mostly elongated and slender. The cytoplasm did not show the pronounced vacuolization found in ordinary scurvy but was often filled with PAS-reactive granules. *Formation of extracellular, often strongly metachromatic, material was noticed in most cases* (Plate IV: 3). The ground substance thus formed had a fibrous appearance and was but poorly stained both with Hale's colloidal iron and by the PAS reagents. Intermingled with this "fibrous" ground substance, however, there was in addition a certain amount of an amorphous variety that stained rather well with the Hale method and faintly with Schiff's reagent (Plate IV: 6).

The effect on the formation of fibrils was not as obvious as the influence on the cellular activity. There is no doubt, however, that an increased formation of reticular fibrils could be observed (Plate IV: 2) when compared to the formation of fibers in ordinary scurvy. Moreover, the fibrils showed a tendency to be orientated, a tendency which was also noticeable among the cellular elements (Plate IV: 4—5).

The ground substance formed in these animals showed on the whole the same histochemical properties as that found in normal animals. The amount produced in this group, it is true, was definitely smaller when compared to the normal animals. Furthermore, the production of the apparently "normal" matrix was not followed by an adequate formation of fibrillar elements. Thus the healing of the skin wound was rather imperfect on the 8th day after operation (Plate I: 5).

No significant effect of hyaluronidase could be found i the granulation tissue surrounding the enzyme-containing elder-pith.

*Effect of cortisone in normal animals: Group VII a.* — In this group a significant depressing effect on the development of normal fibroblasts as well as on the formation of fibrillar elements could be observed (Plate VI: 1, 3, 5). The fibroblasts looked swollen with a vacuolated cytoplasm, thus resembling the cells found in the scorbutic animals (Plate III: 6). They showed a restricted proliferation and were generally separated without any tendency to form a syncytium. The production of histochemically demonstrable ground substance seemed to be significantly depressed, the spaces between the cells being practically non-reactive both to PAS staining and to Hale's colloidal iron, and only in certain areas were they metachromatically stainable with toluidine blue. Treatment with testicular hyaluronidase did not change the histochemical reactivity of the areas concerned. The well-known inhibition of wound healing is clearly demonstrated in Plate I: 4 where a complete failure of repair is evident.

*Effect of adrenalectomy in scorbutic animals: Groups II and V.* — These two groups may be considered together as the findings were on the whole identical in both.

Adrenalectomy apparently aggravated the "scurvy lesions" within the connective tissue elements. The proliferation of abnormal fibroblasts was comparatively moderate, the cells being irregularly distributed without any tendency to form a syncytium. Most of the cells were of a small size with pychnotic nuclei and a scanty amount of cytoplasm. Their ability to organize the blood clots within the wound areas seemed to be highly reduced. On the 8th day after operation there were still large hemorrhages which showed no signs of fibroblasts migrating into them. At that time a few irregular strands of argyrophilic material could be observed between the cells but no reticular fibrils with a normal appearance seemed to be laid down. The cells apparently produced a small amount of water-soluble, non-metachromatic matrix which took a rather faint color with the Hale reagents as well as with the PAS staining. The healing of the skin incision was extremely poor (Plate I: 6).

When *cortisone* was administered to the *scorbutic, adrenalectomized* animals (Group VI) the fibroblasts produced a comparatively large amount of ground substance. On the 5th day after operation a significant amount of Hale-positive material could be seen in the intercellular spaces (Plate V: 1). This material showed an amorphous appearance. By the 8th day fine threads with a high metachromatic potency were spun off the cells (Plate V: 2). This picture strikingly resembles that found in Group IV (Plate IV: 3). At this stage the cells showed a certain orientation and they became uniform in size and shape (Plate V: 3—5). Their production of reticulin was however very low.

*Adrenalectomy performed in normal animals (Group VIII c)* apparently caused a diminished production of metachromatically stainable ground substance. The fibroblasts appeared normal on the whole, but a number of them included a material in the cytoplasm which took a metachromatic color with toluidine blue and which showed a significant PAS-reactivity. Extracellular ground substance could be visualized with the Hale technic within areas where no metachromasia could be observed after staining with toluidine blue. Furthermore, the interfibrillar substance showed a significantly stronger PAS-reactivity than was observable in the granulation tissue of normal animals.

When cortisone was administered to adrenalectomized normal animals (Group VIII c) the reparative processes seemed to be histologically and histochemically indistinguishable from those observed in the sham-operated normal animals (Group VII a).

CHAPTER VII

## Local treatment with ascorbic acid, dehydroascorbic acid and glucuronic acid

16 animals which had been fed the basal diet for 2 weeks or more were used. At the time of operation all these animals showed significant signs of scurvy with swollen knee-joints and loss of appetite. Six animals were treated with pure ascorbic acid dissolved in water at a concentration of 50 mg %. Of the remaining 10 animals 5 were treated with dehydroascorbic acid at the same concentration as that of the ascorbic acid, and the other 5 animals were treated with 0.5 % glucuronic acid lactone dissolved in water.

As has been described above, 4 circular wounds were made in the dorsal skin of each animal. 0.1 ml of the solutions described above were applicated daily to two of the skin wounds whereas pure water was dropped on the other two wounds of the animal. The application of the solutions was performed under sterile precautions. The treatment started in accession to the operation. One animal from each group was killed every other day from the 2nd day after operation. The histotechnical treatment of the wounds was the same as used in the other experiments recorded.

### Results

Of the substances tested only ascorbic acid and dehydroascorbic acid showed any significant effects on the healing processes of the skin wounds. The local application of glucuronic acid induced a very slight formation of metachromatically stained intercellular material within the wound area but no significant effect on the cells or on the production of fibrils was observed (Plate VII: 4).

The effect produced by *locally applied ascorbic acid* was quite significant after 3—4 days of treatment. At that time a moderate amount of metachromatically stained ground substance appeared in the intercellular spaces (Plate VII: 6) and reticular fibrils were laid down in abundance. The PAS reactivity of the ground substance decreased as the metachromatic potency increased. Within the time of observation no collagenous fibers were produced.

*Dehydroascorbic acid* showed an effect quite as pronounced as that caused by ascorbic acid. The intercellular substance produced after 3—4 days' treatment with this agent was of an amorphous character and reacted fairly strongly with the PAS staining. Its metachromatic potency was moderate (Plate VII: 5). The fibroblasts became slender and orientated and there was a strongly increased production of argyrophilic fibrils (Plate VII: 2). No mature collagen was formed during the period of these experiments.

*Comment.* — The quantities of vitamin C applicated on the wounds (0.05 mg/day) did not have any influence on the general ascorbic acid content of the tissues. Moreover, there was a definite difference between the wounds treated with the vitamin and the controls.

According to Bourne (1942) there is disagreement whether vitamin C given locally has any effect on the healing process in wounds of scorbutic animals. The present studies seem to indicate that a certain effect may be achieved by local treatment with ascorbic acid and dehydroascorbic acid i. e. vitamin C. The effect was, however, moderate and observable only after a comparatively long treatment. This result may be interpreted as being indicative of an *indirect* action of vitamin C on the ground substance, possibly mediated through the cellular elements.

CHAPTER VIII

# Discussion

The histochemical findings reported above evidently support the assumption made on the basis of the functional experiments performed that in ascorbic acid deficiency there is a comparatively large production of a ground substance, the physico-chemical properties of which differ from those ordinarily found in normal animals.

In view of the histochemical features exhibited by this abnormal ground substance it seems justified to assume that it is present within the connective tissue in a disaggregated and depolymerized state. In this condition the ground substance evidently lacks metachromatic potency but shows a significant reactivity to the periodic acid-Schiff reaction, probably due to release of reactive glycol groups.

The ground substance produced within a wound area may possibly be different from that existing in the intact connective tissues. It seems probable, however, that the cells responsible for the production of the ground substance form a material that possesses the same general physico-chemical properties wherever it is "secreted". The delicate problem concerning the physico-chemical features of the ground substance-precursor(s) passing the cell membrane thus presents itself. At the present stage of our knowledge, there can be no contributions to the understanding of this problem but new speculations.

The disintegrated ground substance supposed to be present in scurvy may either represent the form which is secreted by the cells or may be the product of a depolymerization outside the cells. The first of these two possibilities is in agreement with the assumption made by Wolbach and Howe (1926) that in scurvy there fails an agent common to all supporting tissues which is responsible for the setting, fibrillation, or jelling of a product which would otherwise remain liquid. The classic description of the healing process made by Bensley (1934) may also be mentioned in this connection. She described the cycle common to all healing processes in connective tissue as *edema, gelation, and fiber formation.* This pattern of healing has been observed and described by many research workers after Bensley. In the case of scurvy the whole process may be assumed to have stopped at the first stage, edema.

It may be that in scurvy the rapidly multiplying, mesenchyme-like fibroblasts are unable to produce acid mucopolysaccharides of a normal degree of polymerization. *Scurvy might thus be characterized as a state of overproduction of incompletely polymerized ground substance.* The rapid action of ascorbic acid on granulation tissue in scurvy (Penney and Balfour, 1949) implies a depression of the rate of multiplication of the fibroblasts and a concomitant gelation of the ground substance. If this action is due to an inhibition of a hypothetically high activity of hyaluronidase outside the cells, or to a normalizing effect on the mechanism of the mucopolysaccharide production within the cells is a question which cannot be answered at present.

The significant effect of cortisone seems to be related to the second stage of the cycle, gelation. The mechanism of this action is obscure.

Cortisone seems to inhibit the development of fibroblasts, and in this way the hormone affects the processes of repair (Ragan, 1952). Once the fibroblast is laid down, in contradistinction to scurvy, collagen and ground substance is deposited whereas in scurvy the effect is on the function of the fibroblast after this cell has made its appearance. The effect of cortisone on the cells preceding the fibroblasts can be produced by local application of the hormone (Castor and Baker, 1950).

According to the present experiments, the inhibitory effect of cortisone on the regenerating processes in connective tissue found in normal individuals cannot be observed in scurvy. This may be explained by the fact that scurvy involves a significantly increased fibroblastic proliferation and thus counteracts the depressing effect of cortisone on the development of these cells. When fibroblasts are present already, the action of cortisone seems to be on the "gelation mechanism". Apparently the hormone in some way induces the polymerization or aggregation of the water-soluble, more or less depolymerized ground substance already present intercellularly, or perhaps it prevents the depolymerization of newly formed highly polymerized ground substance by acting as a hyaluronidase inhibitor (Hench *et al.,* 1949).

Whatever the exact mechanism of cortisone as a "gelation factor" in scurvy, it may be concluded that an adequate amount of this hormone seems to be indispensable for an adequate function of connective tissue ground substance. The "semi-scorbutic" condition produced by adrenalectomy in guinea pigs confirms such a suggestion.

# General Summary

## Part I

The histochemical properties of purified samples of hyaluronic acid and chondroitin sulfuric acid as well as of commercial heparin and native samples of vitreous humor and synovial fluid have been investigated. The histochemical methods studied were the metachromatic staining with thiazin dyes, the staining with Schiff's leucofuchsin after oxidation with periodic acid or sodium bismuthate, and Hale's colloidal iron-ferrocyanide method.

According to the results obtained in vitro the following conclusions were made regarding the reactivity of hyaluronic acid chondroitin sulfuric acid towards the various reagents:

1. When highly polymerized, hyaluronic acid may possess a significant metachromatic potency, the demonstration of which depends on the purity of the dye used.
2. Partly depolymerized hyaluronic acid in a purified form reacts very weakly or not at all with Schiff's reagent even after prolonged oxidation with periodic acid.
3. Hyaluronic acid has a certain affinity to colloidal iron; this affinity is retained even when the mucopolysaccharide is depolymerized.
4. When "sufficiently" depolymerized, hyaluronic acid may show a significant reactivity to Schiff's reagent after an adequate oxidation with periodic acid or sodium bismuthate.
5. Hyaluronic acid-protein complexes as found in synovial fluid may show a strong metachromasia, a certain affinity to colloidal iron and a significant reactivity towards the periodic acid-Schiff reagents.
6. The reactivity of hyaluronic acid to the various reagents tested seems to vary not only with the degree of polymerization but also with the origin of the preparation.
7. Chondroitin sulfuric acid prepared from hyaline cartilage shows a strong chromotropic potency, a significant affinity to colloidal iron and a certain reactivity to Schiff's leucofuchsin even after a moderate oxidation with periodic acid.
8. Commercial heparin possesses a strong metachromatic property and may be demonstrated by Hale's colloidal iron or with the periodic acid-Schiff technic. The significant reactivity towards Schiff's reagent

after oxidation with periodic acid may be due to the content of monosulfated heparin.

9. The histochemical implications of the reactions observed in vitro are discussed.

## Part II

The organization of the connective tissue ground substance in ascorbic acid deficiency induced in growing female guinea pigs has been studied with the aid of functional, chemical and histochemical tests. The influence of cortisone on the actual state of the ground substance in scurvy has been investigated.

According to the data obtained with the various methods used the following conclusions have been drawn:

1. An increased amount of ground substance mucopolysaccharides is found in scurvy; the concentration of hexosamine within the skin is significantly increased in comparison to normal animals.

2. The ground substance complex is present in an abnormal state of organization, showing an increased solubility in water and a significantly lowered resistance towards penetrating agents; the dermal spread of Evans blue is increased like the velocity of flow of Ringer's solution through isolated muscle fascia.

3. The water-binding capacity of the skin is increased.

4. At repair after wounds a depolymerized, water-soluble ground substance is found within the granulation tissue. This ground substance does not show any metachromatic potency but reacts with Schiff's reagent after periodic acid and with Hale's colloidal iron. The fibroblasts contain large amounts of Schiff-reactive material.

5. Adrenalectomy aggravates the symptoms of scurvy and seems to inhibit the cellular activity in granulation tissue.

6. Cortisone induces a marked gelation of the matrix. The dermal spreading of Evans blue shows a tendency to be depressed; the amount of hexosamine in the skin decreases and the dermal content of water is lowered as compared to untreated scorbutic animals. The histochemical properties of the ground substance change at treatment with cortisone in scurvy: the matrix becomes metachromatic and its reactivity to the periodic acid-Schiff staining seems to be diminished.

7. Local treatment of skin wounds with ascorbic acid and dehydroascorbic acid were effective in causing a gelation of the ground substance when the treatment is extended over several days.

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Plates I—VII

## PLATE I

Explanation of figures.

1. Skin wound of a normal guinea pig 8 days after operation. Wound area filled up with orientated connective tissue. Azan. 80 ×.

2. Skin wound from a partially ascorbic acid-depleted animal (Group I). Moderate degree of fibroplasia and degeneration of the mature collagen Azan. 80 ×.

3. Skin wound of scorbutic animal 8 days after operation. Large dilated vessels and non-organized hemorrhages are seen in the wound area. Very low degree of fibrillogenesis. Azan. 80 ×.

4. Skin wound of a normal guinea pig treated with cortisone (5 mg/100 g body weight once a day for 8 days after operation). Practically no signs of migration of fibroblasts into the wound area. No formation of collagen. Azan. 80 ×.

5. Skin wound from a scorbutic animal treated with cortisone (dosage: see above) 8 days after operation. Marked fibroplasia with well orientated reticulin fibrils. Azan. 80 ×.

6. Skin wound from a scorbutic adrenalectomized animal 8 days after operation. The wound area is filled upp with degenerated fibrin, dilated vessels and a small amount of abnormal reticulin. Azan. 80 ×.

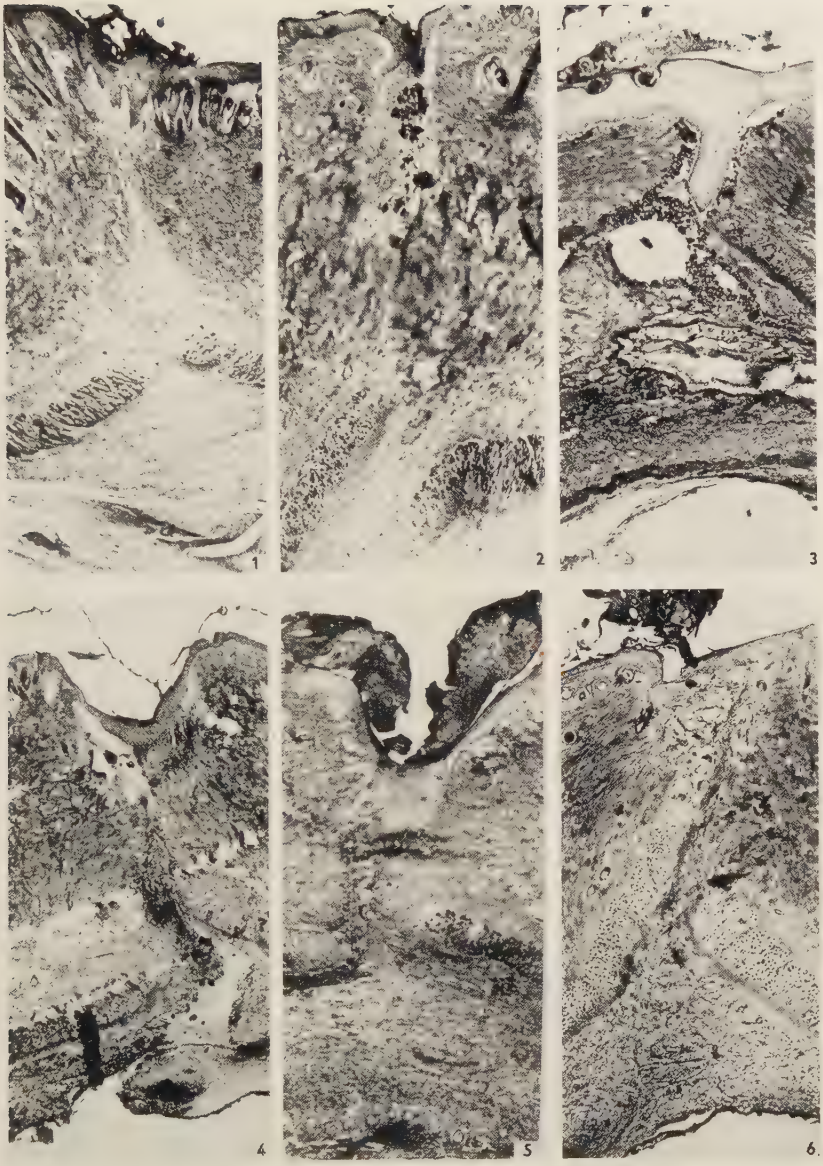



Plate I: 1—6.

## PLATE II

Explanation of figures.

1. Granulation tissue surrounding an implanted elder-pith. 5 days after operation. The fibroblasts are uniform in size and shape and well orientated. Azan. 160 ×.

2. The same tissue area as in 1) impregnated with silver. Orientated reticulin fibrils. 160 ×.

3. Muscle wound 2 days after operation. Migrating fibroblasts showing varying shapes and sizes. Basophilic inclusions within the cytoplasm. the cells are embedded in an mainly amorphously precipitated ground substance showing a strong metachromasia. Tol. bl. 800 ×.

4. Normal fibroblast found within granulation tissue from a muscle wound 5 days after operation. Scanty cytoplasm bordered by spun off threads which show a distinct metachromasia. The surrounding ground substance partly amorphous and partly of a fibrous character. Tol. bl. About 1 800 ×.

5. Normal granulation tissue surrounding an implanted elder-pith 8 days after operation. The fibroblasts are uniformly shaped surrounded by a ground substance showing a fibrous structure and a strong metachromasia. Tol. bl. 400 ×.

6. The same tissue area as in 5) stained with the PAS method. The reticular and collageneous fibers show a positive reaction. The interfibrillar substance practically unstained. 400 ×.

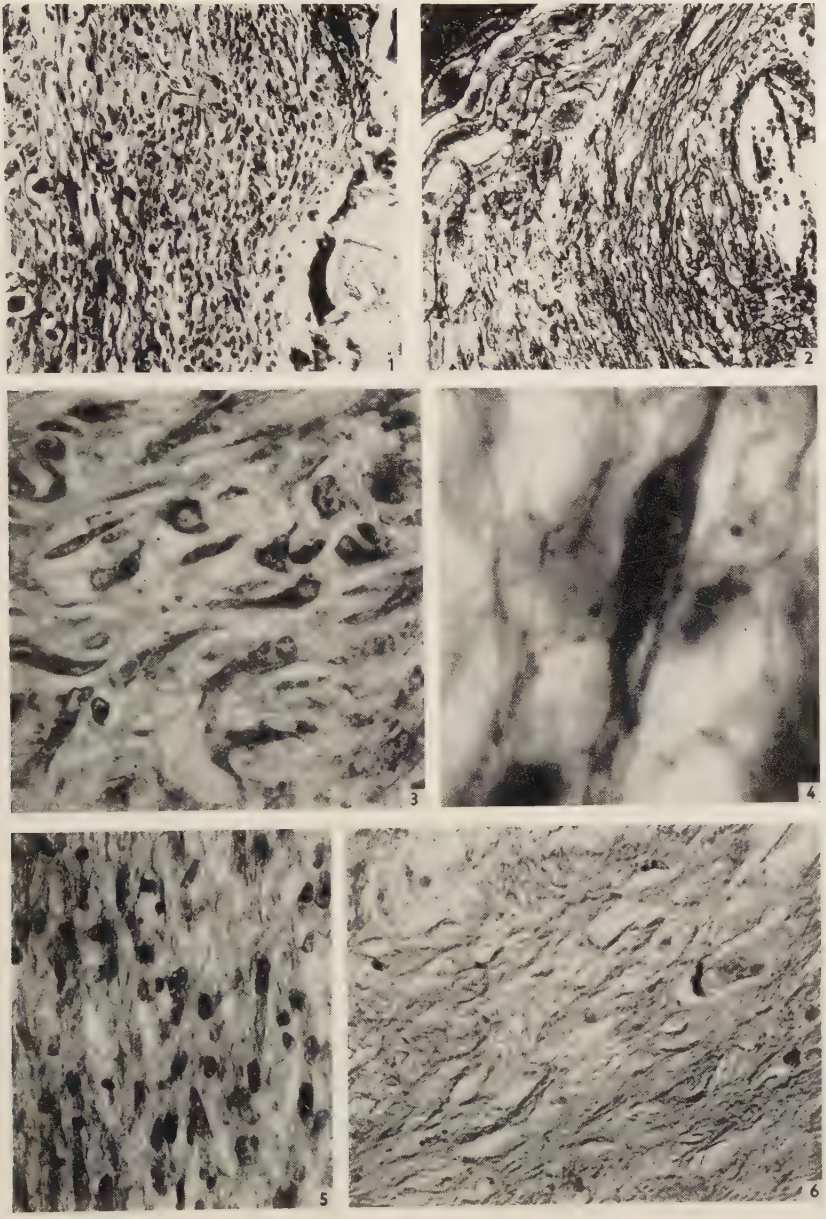



Plate II: 1—6.

## PLATE III

Explanation of figures.

1. Elder-pith surrounded by a scanty foreign body granulation tissue. Scorbutic animal. 8 days after operation. Azan. 8×.

2. Detail from 1) showing degenerated fibrin, hemorrhages and no signs of collagen. Azan. 160×.

3. The same tissue stained with silver. The clumsy reticular fibrils are clearly shown. 160×.

4—6. Abnormal fibroblasts in a granulating muscle wound from a scorbutic guinea pig 5 days after operation. The pronounced variation in size and form is readily seen. The cytoplasm is vacuolated and the nuclei heavily stained. Fig. 4 shows extracellular areas which react with the PAS-reagents exhibiting a granular appearance. Fig. 5 demonstrates Hale-positive extracellular substance whereas toluidine blue shows no metachromatic reaction in the intercellular spaces (Fig. 6). As seen in fig. 4 the cytoplasm of the cells often show PAS-positive inclusions. 800×.

7. Granulation tissue from a scorbutic guinea pig 8 days after surgical intervention. Combined Hale-PAS staining. An abundancy of Schiff-positive granules within the cytoplasm of the cells which are surrounded by a Hale-positive, amorphous ground substance. 800×.

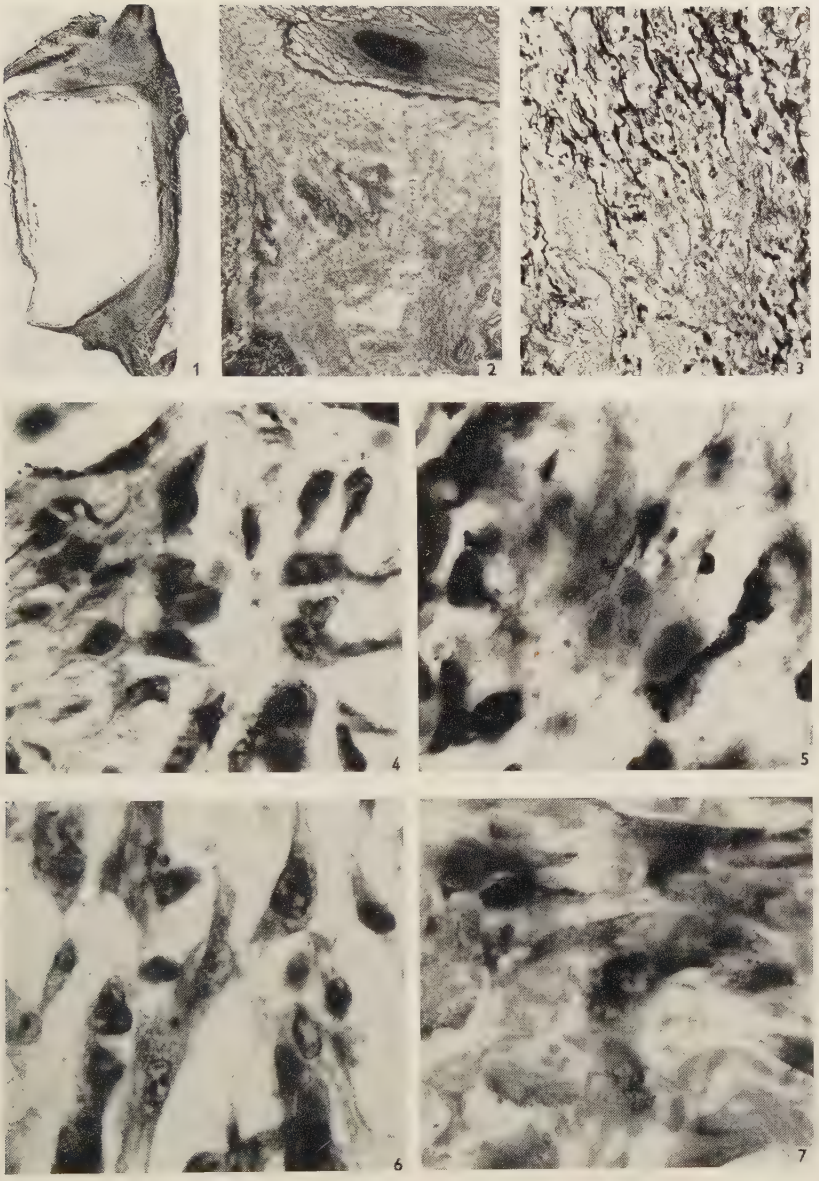



Plate III: 1—7.

## PLATE IV

Explanation of figures.

1. Granulation tissue surrounding an implanted elder-pith plate. Scorbutic guinea pig treated with cortisone (5 mg/100 g b. w./day) for 8 days after operation. Marked fibroblastic activity but scanty amount of fibrillar elements. Streaks of degenerated fibrin are seen. Azan. 80 ×.

2. The same tissue area as 1) impregnated with Gomori's silver technic. An abundancy of irregular reticulin fibrils showing a certain attempt of orientation. 160 ×.

3. Fibroblasts showing a pronounced metachromasia within the cytoplasm embedded in a fibrous ground substance with strong metachromatic potency. Scorbutic, cortisone-treated animal 2 days after operation. 1 200 ×.

4—5. Elongated fibroblasts showing a certain orientation. Outside the cells there are patches of metachromatically stained ground substance. Scorbutic, cortisone-treated animal 5 days after operation. Tol. bl. 800 ×.

6. Elongated, orientated fibroblasts showing pronounced inclusions of Schiff-positive granules in their cytoplasm. Surrounding ground substance faintly PAS-reactive. Scorbutic animal treated with cortisone 8 days after operation. 400 ×.

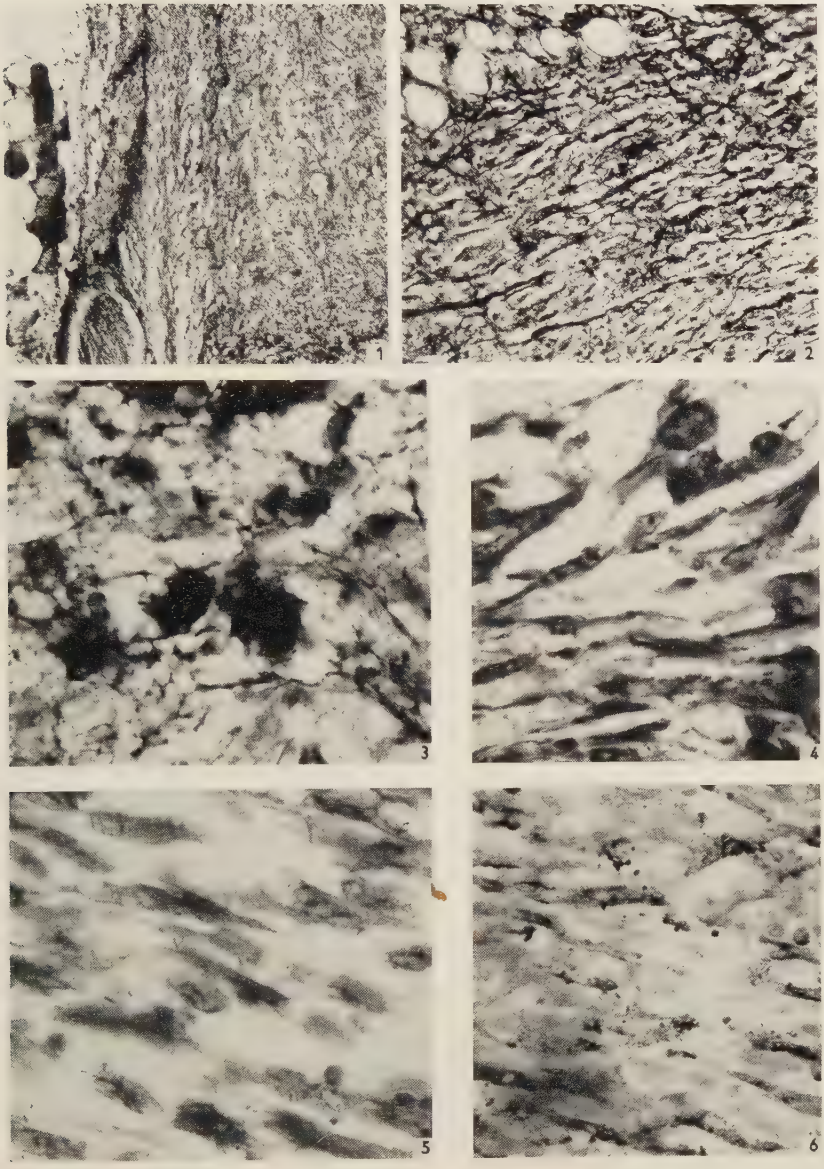



Plate IV: 1—6.

## PLATE V

Explanation of figures.

1. Granulation tissue from muscle wound area of a scorbutic, adrenalectomized animal treated with cortisone 5 days after operation, The amorphous, rather strongly Hale-positive ground substance is well demonstrable. 800 ×.

2. An animal from the same experimental group which has been treated for 8 days after operation with cortisone. The appearance of a fibrous ground substance is evident. Beside there is a certain amount of amorphous matrix to be seen. Tol. bl. 1 200 ×.

3—5. Adjacent sections of granulation tissue from the same animal as in 2). The fibroblasts show a certain orientation and uniformity in size and shape. There is a rather low amount of ground substance visible within these figures (3: stained with combined Hale-PAS; 4: stained with tol. bl.; 5: stained with Hale's reagents). 160 ×.

6. Granulation tissue from a normal guinea pig showing the syncytial arrangement of the fibroblasts and the fibrous structure of the ground substance. 5 days after operation. Hale's method. 800 ×.

7. An adjacent section stained with tol. bl. 800 ×.

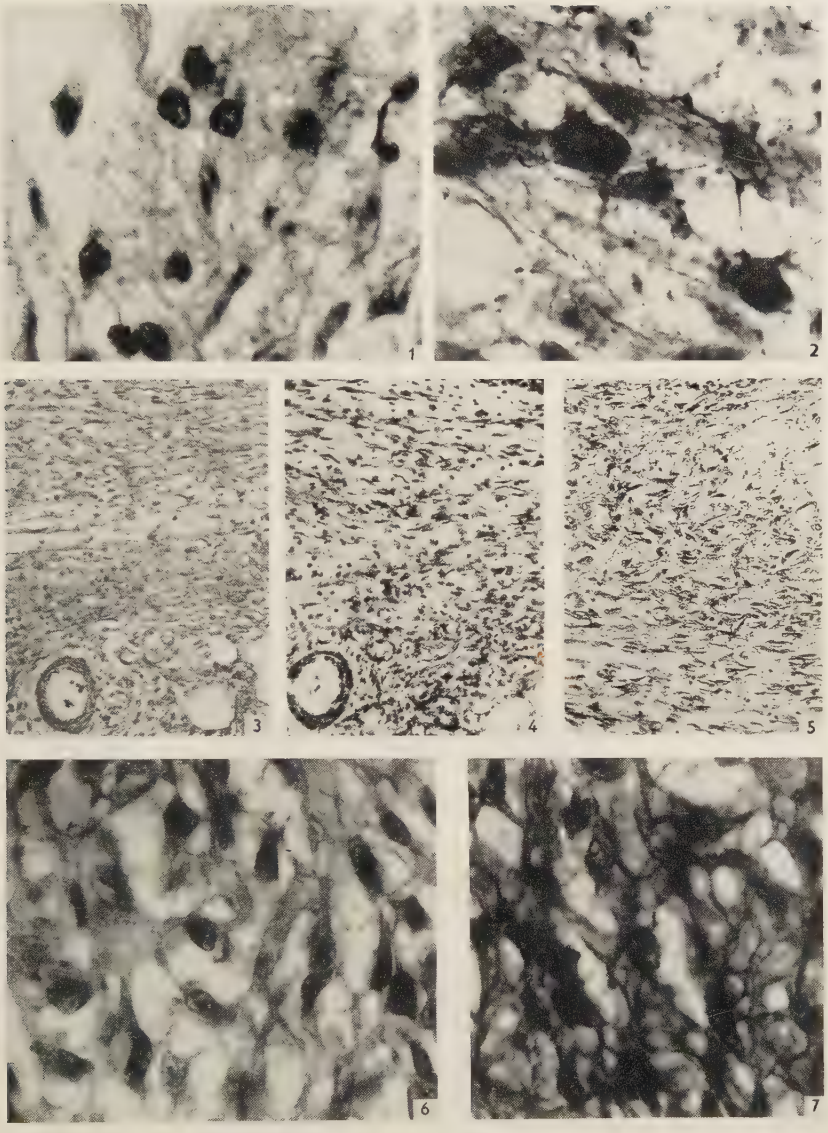



Plate V: 1—7.

## PLATE VI

Explanation of figures.

1. Granulation tissue from normal, cortisone-treated guinea pig showing a chaotic arrangement of clumsy reticulin intermingled with fibrin. 8 days after operation. Azan. 160 ×.

2. A similar section from an adrenalectomized, cortisone-treated animal showing a more normal appearance of the fibrils. Silver. 160 ×.

3. From the same animal as 1). The abundant cytoplasm of the fibroblasts shows a vacuolated appearance with same metachromatic inclusions. Practically no ground substance is stained in this section. Tol. bl. 1 200 ×.

4. The same animal as 2). A few small fibroblasts are seen embedded in the metachromatic stained ground substance. Tol. bl. 1 200 ×.

5. The same animal as 1). The vacuolated fibroblasts are clearly seen. With Hale's colloidal iron a scanty amount of granular extracellular substance is visible. Within the cytoplasm of the cells there are Schiff-reactive granules visible. Hale-PAS. 1 200 ×.

6. The same animal as 2). The combined Hale-PAS technic reveals an abundancy of extracellular ground substance of a granular or fibrous structure. 1 200 ×.

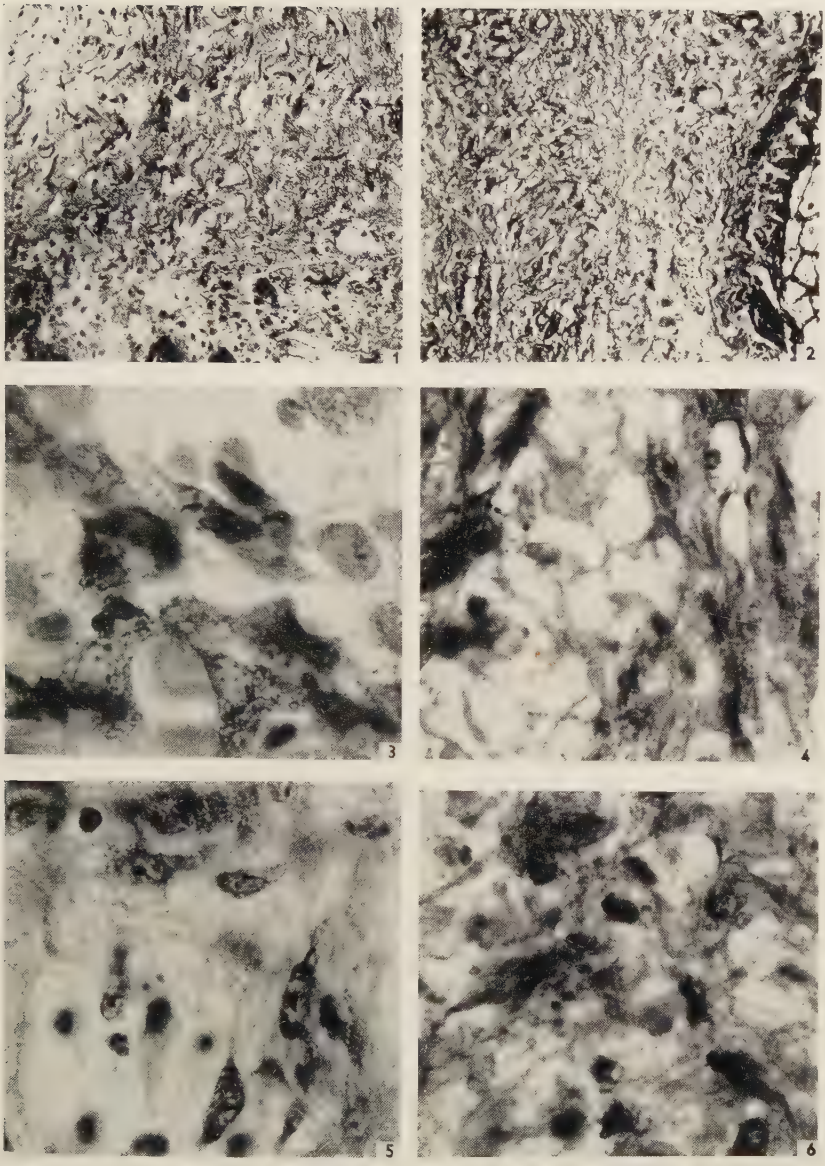


Plate VI: 1—6.

## PLATE VII

Explanation of figures.

1. The edge of a circular skin wound made in a scorbutic animal. 6 days after surgical intervention. No metachromasia visible. Tol. bl. 80×.

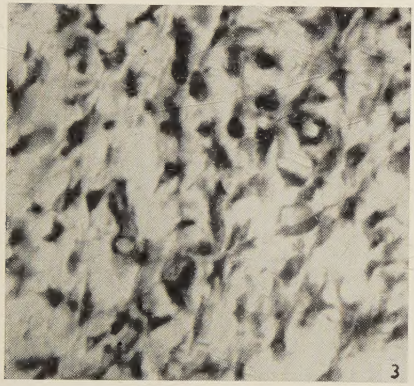
2. A similar section from a wound locally treated with dehydroascorbic acid. Note the excessive formation of fibrils. The ground substance showed a strong metachromasia. Tol. bl. 80×.

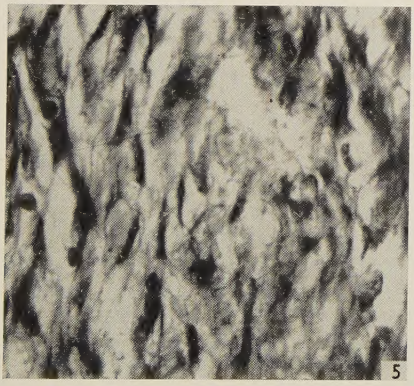
3. Detail from the same tissue area as in 1). A marked fibroblastic proliferation but no metachromatically stainable ground substance. Tol. bl. 500×.

4. Granulation tissue from a skin wound locally treated with glucuronic acid for 4 days after operation. Scorbutic animal. A slight metachromatic reaction is visible within the ground substance which is precipitated as amorphous floccules in the section. Tol. bl. 500×.

5. Detail from the same tissue area as seen in Fig. 2. The fibroblasts look normal and are embedded in a significant amount of metachromatically stained ground substance. Tol. bl. 500×.

6. Granulation tissue from a skin wound of a scorbutic guinea pig. Local treatment of the wound with ascorbic acid for 4 days. The cellular elements show marked varieties in size and shape. The ground substance is well developed and shows a mainly fibrous appearance. Tol. bl. 500×.





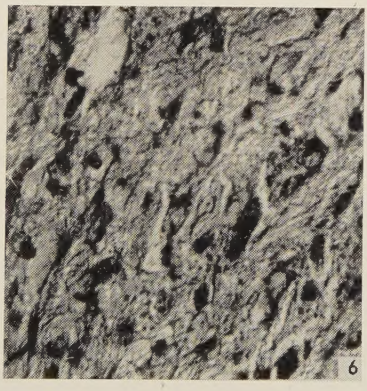




PLATE VII: 1—6